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“Degradación de cortactina en células endoteliales por proteasas de serina de neutrófilos: un nuevo mecanismo específico de neutrófilos para transmigrar”

T E S I S

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“Cortactin degradation in endothelial cells by neutrophil serine proteases: a new neutrophil-specific mechanism to transmigrate”

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ABBREVIATIONS

AAT	Alpha-1-antitrypsin
ABP	Actin binding protein
ACT	Alpha1-antichymotrypsin
AEBSF	4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride
AJ	Adherent junctions
ANCA	Anti-neutrophil cytoplasmic autoantibodies
Arp 2/3	Actin-related protein 1/2
BM	Bone marrow
BM	Basement membrane
CG	Cathepsin G
CTSC	Cathepsin C
DMSO	Dimethyl sulfoxide
DPPI	Dipeptidyl peptidase I
EC	Endothelial cells
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ENDS	Elongated neutrophil derived structures
ERM	Ezrin, radixin, moesin
EV	Extracellular vesicle
F-actin	Filamentous actin
FBS	Fetal Bovine Serum
fMLP	N-Formyl-methionyl-leucyl-phenylalanine
G-CSF	Granulocyte colony-stimulating factor
HRP	Horse-radish peroxidase
HUVEC	Human umbilical vein endotelial cells
I/R	Ischaemia reperfusion
ICAM-1	Intracellular adhesion molecule -1
IFN-γ	Interferon-gamma
IgG	immunoglobulin G
IL-18	Interleukin-18
IL-1β	Interleukin-1-beta

IL-6	Interleukin-6
IL-8	Interleukin-8
IVM	Intravital microscopy
JAMs	Junctional adhesion molecules
KD	Knock down
LFA-1	Lymphocyte-function associated antigen 1
LIMK	LIM kinase
LPS	Lipopolysaccharide
LTB4	Leukotriene B4
Mac-1	Macrophage antigen 1
MLC	Myosin light chain
MMP	Metalloproteinase
MNEI	Monocyte neutrophil elastase inhibitor
MPO	Myeloperoxidase
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Neutrophil Elastase
NETs	Neutrophils Extracellular Traps
NFP	Nucleation-promoting factors
NF-κB	Nuclear factor kappa B
NSP	Neutrophil serine proteases
NSP4	Neutrophil serine protease 4
PAF	Platelet activating factor
PAK	p21-activated kinase
PAR	Protease-activated receptor
PBS	Phosphate-buffered saline
PCV	Post-capillary venules
PECAM-1	Platelet endothelial cell adhesion molecule
PFA	Paraformaldehyde
PMN	Polymorphonuclear cells
PMSF	Phenylmethylsulfonyl fluoride
PR3	Proteinase 3
P-selectin	Platelet-Selectin
PSGL-1	P-selectin glycoprotein ligand-1

PYK2	Protein tyrosine kinase-2
ROCK	Rho kinase
ROS	Reactive oxygen species
RT	Room Temperature
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLPI	Secretory leukocyte proteinase inhibitor
SV	Secretory vesicles
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline-Tween
TEM	Trans-endothelial migration
TGF-β	Transforming growth factor-beta
TJ	Tight junctions
TNFα	Tumor Necrosis Factor-alpha
TPM	Trans-pericyte migration
VCAM-1	Vascular cell adhesion molecule-1
VE-cadherin	Vascular endothelial-cadherin
VE-PTP	Vascular-endothelial protein tyrosine phosphatase
WT	Wild type
ZO	Zonula occludens
α2M	α2-macroglobulin
α-SMA	alpha-smooth muscle actin

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RESUMEN

La extravasación de neutrófilos dentro de tejidos inflamados es esencial para combatir las infecciones bacterianas, virales y parasitarias. Sin embargo, la infiltración excesiva de neutrófilos se asocia con daño agudo de tejidos. Por lo tanto, una mejor comprensión de los mecanismos que regulan la extravasación de neutrófilos puede conducir a mejores estrategias terapéuticas que equilibren el tráfico de neutrófilos a niveles protectores sin inducir daño tisular. Para llegar al sitio de la inflamación, los neutrófilos deben cruzar las vénulas post-capilares. El endotelio venular remodela dinámicamente su citoesqueleto de actina para regular la diapédesis de neutrófilos. Sin embargo, los mecanismos exactos que controlan el reordenamiento del citoesqueleto de actina endotelial durante este proceso siguen siendo poco conocidos. En este proyecto, investigamos cómo la dinámica de la proteína de unión a actina endotelial cortactina regula la extravasación de neutrófilos mediante una combinación de modelos *in vivo* e *in vitro*. Encontramos que cortactina sufre degradación proteolítica en las vénulas post-capilares cremastéricas durante la inflamación local aguda. Por otro lado, una porción de cortactina translocada a los sitios donde se adhieren los neutrófilos a las células endoteliales está protegida de la degradación. De manera importante, la degradación de la cortactina se inhibió *in vitro* cuando los neutrófilos se trataron con el inhibidor farmacológico de las serina-proteasas AEBSF y PMSF, e *in vivo* cuando los ratones se sometieron a una terapia de aumento de α1-antitripsina (AAT), un inhibidor endógeno de las serina-proteasas de los neutrófilos. Usando estas estrategias, demostramos que la cortactina endotelial es degradada por serina-proteasas de neutrófilos (NSP) que se transfieren a las células endoteliales durante la adhesión de neutrófilos por un mecanismo aún desconocido. Además, el tratamiento con AEBSF y PMSF, así como la terapia de aumento de AAT, resultó en una reducción de la extravasación de neutrófilos *in vitro* e *in vivo*, respectivamente. Mecanisticamente, la degradación de la cortactina citosólica se asoció con una mayor formación de fibras de estrés de actina contráctiles, lo que probablemente aumenta la apertura de los contactos endoteliales y, por lo tanto, facilita la diapédesis paracelular de neutrófilos. En conjunto, identificamos la degradación de cortactina por NSP como un mecanismo específico de neutrófilos para facilitar la diapédesis de neutrófilos a través de una mayor contractilidad de las fibras de estrés de actina. Entonces, la terapia de aumento de AAT que inhibe la degradación de cortactina podría servir como nueva estrategia terapéutica para tratar enfermedades inflamatorias.

ABSTRACT

Neutrophil extravasation into inflamed tissues is essential to fight bacterial, viral, and parasitic infections. However, excessive neutrophil infiltration is associated with acute tissue damage. Therefore, a better understanding of the mechanisms regulating neutrophil extravasation may lead to better therapeutic strategies that balance neutrophil trafficking to protective levels without inducing tissue damage. To reach the site of inflammation, neutrophils must cross post-capillary venules. The venular endothelium dynamically remodels its actin cytoskeleton to support neutrophil diapedesis. However, the exact mechanisms that control endothelial actin cytoskeleton rearrangements during this process remain poorly understood. Here, we investigated how the endothelial actin-binding protein cortactin regulates neutrophil extravasation by a combination of *in vivo* and *in vitro* approaches. We found that cortactin is a target for proteolytic degradation in cremasteric post-capillary venules during acute local inflammation. However, a pool of cortactin that was translocated to the docking sites where neutrophils adhere to the endothelium is protected from degradation. Cortactin degradation was inhibited *in vitro* when neutrophils were treated with the pharmacological serine protease inhibitors AEBSF and PMSF, and *in vivo* when mice were subjected to α1-antitrypsin (AAT) augmentation therapy, an endogenous neutrophil serine protease inhibitor. Using these strategies, we demonstrated that endothelial cortactin is degraded by neutrophil serine proteases (NSP) that are transferred to endothelial cells during the adhesion of neutrophils by yet unknown mechanisms. Moreover, AEBSF and PMSF treatment as well as AAT augmentation therapy resulted in reduced neutrophil extravasation *in vitro* and *in vivo* respectively. Mechanistically, the degradation of cytosolic cortactin was associated with increased formation of contractile actin-stress fiber, which likely increased the opening of endothelial contacts, and thus facilitated neutrophil paracellular diapedesis. Collectively, we identified cortactin degradation by NSP as a neutrophil-specific mechanism to facilitate neutrophil diapedesis via increased contractility of actin stress fiber. AAT augmentation therapy that inhibits cortactin degradation could therefore be a promising novel pharmaceutical strategy to treat inflammatory diseases characterized by excessive neutrophil recruitment.

I. INTRODUCTION

1.1. Overview of the inflammatory response

The inflammatory response includes a variety of physiological processes that are coordinated mostly by cytokines, plasma proteins, and immune cells in order to combat infections and resolve tissue injuries [1,2]. The inflammation cascade begins with the recognition of pathogens and damaged cells by innate immune receptors. This initial recognition is mediated by tissue-resident macrophages, mast cells and dendritic cells, which trigger the production of a variety of inflammatory mediators, including several cytokines such as interleukin-1-beta (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) and a variety of chemokines such as IL-8 (CXCL1 in the mouse) and CCL2 [3,4]. The main and most immediate effects of these mediators include the activation of the vascular endothelium leading to increased vascular permeability and plasma protein leakage (complement, antibodies, and acute phase reactants) to extravascular tissue, and the recruitment of circulating leukocytes to the site of inflammation [2,5]. During this process, endothelial cells that line the inner aspect of blood vessel walls express adhesion molecules that, together with the released chemokines, capture, activate leukocytes on the surface of the activated endothelium so that they can eventually cross the vascular wall into the inflamed tissue [6]. In this context, neutrophils are the first leukocytes to reach the sites of inflammation and mediate the onset of the effector response [6]. Once arrived at the inflamed tissue sites, these cells directly eliminate the pathogens through phagocytosis and a combination of oxidative and non-oxidative mechanisms [7–9]. Finally, after resolution of inflammation, neutrophils undergo apoptosis and are phagocytosed by resident macrophages and dendritic cells [10].

1.2. Neutrophils are key players of inflammation

Neutrophils or polymorphonuclear leukocytes (PMN) are a population of short-lived myeloid cells specialized in promoting inflammatory responses and combating infections by bacteria, fungi, parasites and viruses [11]. Their function is indispensable for the host defense as demonstrated by the worst prognosis of patients with acquired or congenital neutropenia during infection[12–14]. Neutrophils

are mainly characterized by its multilobed segmented nucleus, and their high content of cytoplasmic granules, which store an array of anti-microbial peptides, cytotoxic proteases and adhesion molecules. They are produced during granulopoiesis in the bone marrow (BM) and are released to the peripheral blood when G-CSF induces the complete maturation of neutrophils. G-CSF upregulates the expression of CXCR2 on neutrophils, and increases the secretion of CXCL2, CXCL1 and IL-8 (in humans) in BM endothelial cells, which determines the mobilization of mature neutrophils ($CXCR2^{+}$) from the BM to the blood circulation. In systemic circulation, mature neutrophils represent approximately 15% of total leukocytes in mice, whereas in humans they reach up to 50-70% under steady-state conditions; however, this can increase during infections [10,11,15,16].

Neutrophils constantly circulate in blood vessels under basal conditions waiting for the signals to be recruited to the site of inflammation. On their way towards an infection or tissue injury, neutrophils undergo intravascular priming by recognizing cytokines such as TNF-α IFN-γ, IL-1β and IL-18; chemoattractants such as IL-8, CXCL1/2, C5a, LTB4 and PAF; and adhesion molecules expressed at the lumen side of vascular endothelial cells close to the site of inflammation including ICAM-1, E-selectin and P-selectin [17]. Likewise, the interaction of neutrophils with endothelial cells induces the release of granular contents, particularly matrix metalloproeases and other pro-inflammatory mediators, which promote neutrophil trans-endothelial migration in the venules [17]. Once emigrated into the inflamed extravascular tissue, neutrophils are fully activated by additional pro-inflammatory cytokines such as TNF-α, IL-1β and IL-8, and the recognition of bacterial or viral molecules such as LPS, fMLP, or opsonized bacteria. After full activation, neutrophils exert various mechanisms to efficiently clear invading microorganisms (Figure 1). Neutrophils engulf the pathogens into a phagosome to subsequently degrade them. Then, the NADPH oxidase complex is assembled on the phagosome membrane, and on the plasma membrane to generate large amounts of reactive oxygen species (ROS) inside the phagosome and in the extracellular space [17]. Furthermore, the neutrophil granules containing proteolytic enzymes and anti-microbial peptides are either fused with the membrane of the phagosome to induce its maturation to a phagolysosome causing degradation of the sequestered pathogen, or they are released into the extracellular space by

exocytosis to kill extracellular pathogens [16,17]. When encountering pathogens, neutrophils can also expel web-like structures of decondensed chromatin fibers containing histones and granular enzymes known as neutrophil extracellular traps (NETs). NETs are released into the extravascular space and bind to viruses, bacteria, fungi and parasites to trap them and to block their dissemination to distant organs, as well as to facilitate phagocytosis. NETs are mainly released after recognition of pathogens and pathogen-derived molecules and are accompanied by the cell death in a process called classical NETosis. However, neutrophils can also extrude NETs without affecting viability and effector functions in a process known as “vital NETosis”. Vital NETosis has also been observed after pathogen recognition and NETs are then released by transport of nuclear DNA inside of vesicles that pass through the cytoplasm until being released into the extracellular space. However, the molecular mechanisms that regulates the release via one or the other NETosis pathway is not yet well defined [18–20].

Since these potent effectors act on pathogens but also host tissue, they can cause collateral tissue damage. Thus, uncontrolled, and prolonged neutrophil-mediated immune responses can also contribute to acute and/or chronic inflammatory disorders [9,17,21]. As such, it is now well accepted that excessive neutrophil recruitment and neutrophil-dependent vascular leakage are part of the pathological mechanisms that lead to tissue damage and/or organ dysfunction in cardiovascular diseases, chronic inflammatory lung diseases, and sepsis [21,22]. For this reason, a detailed understanding of the cellular and molecular mechanisms that regulate neutrophil extravasation and neutrophils responses is required.

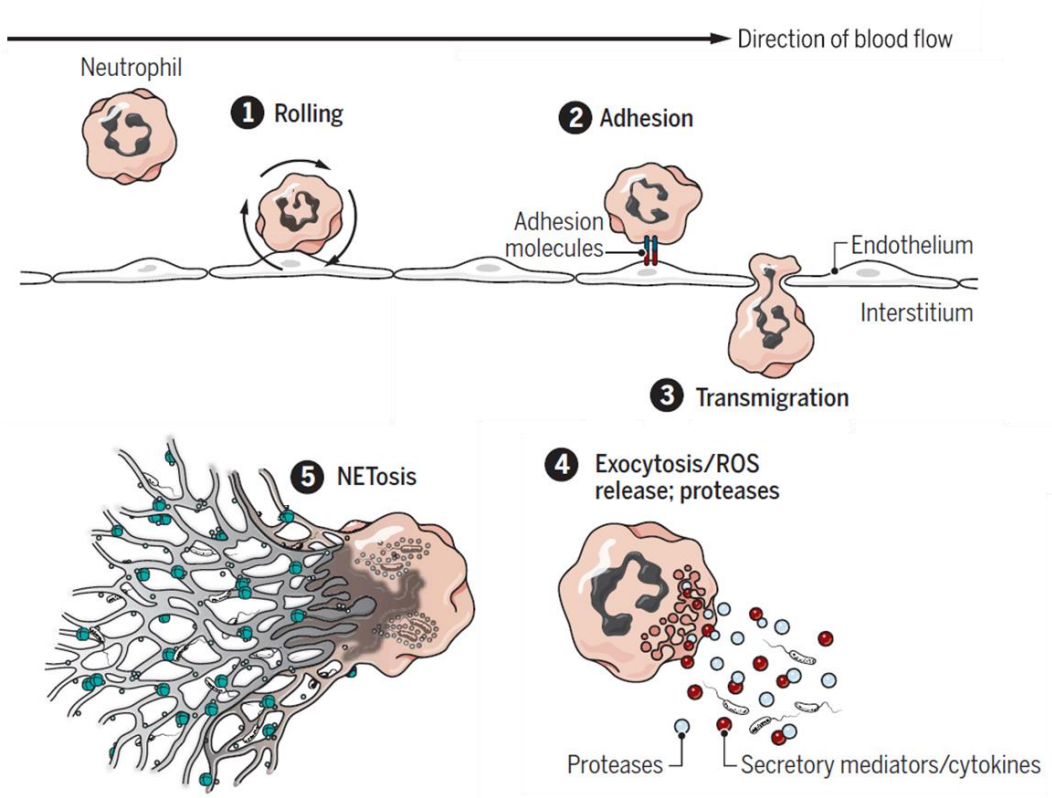

Figure 1. Neutrophils in infection and inflammation. In response to infection, neutrophils are recruited from the blood stream to the inflamed tissue. **(1 and 2)** Neutrophils interact with the lumen of activated venular endothelium via adhesion molecules that mediate the rolling and adhesion at the site of inflammation. **(3)** Neutrophils then leave the venules by transmigrating through the venular wall into the inflamed extravascular tissue. **(4 and 5)** In the interstitium, neutrophils display their defense mechanisms that include the internalization of pathogens for intracellular killing, the release of proteases and ROS, to kill extracellular pathogens, and the formation of NETs that help to trap pathogens [11].

1.3. Neutrophil recruitment through different venular components to sites of inflammation

Neutrophil extravasation from the blood stream into inflamed tissues represent an early line of host defense against infection [6]. This recruitment occurs through a series of complex cellular and molecular events that are coordinated by cytokines, gradients of chemokines and an array of cell adhesion molecules, such as integrins and selectins in both neutrophils and cells of the venular wall [23,24]. Briefly, recruitment is initiated by capture of circulating neutrophils and rolling

along the vascular endothelium mediated by weak and reversible adhesive interactions. This is followed by chemokine-mediated activation of leukocytes leading to firm adhesion, crawling and eventually transmigration through the vessel wall. However, to completely emigrate from the blood vessel, neutrophils need to cross the three different venular barriers: endothelial cells (EC), the vascular basement membrane, and the pericyte layer [25,26]. Although it is well known that neutrophils possess a highly efficient migratory capacity, many seminal works have well described that endothelial cells also actively support leukocytes during extravasation as described in detail below.

1.3.1. The endothelial barrier is regulated by actin cytoskeleton dynamics

Neutrophils use post-capillary venules as the primary site of transmigration [27]. This venular endothelium is the first barrier for emigrating neutrophils. It consists of a confluent monolayer of endothelial cells adjoined by junctional adhesive structures connecting adjacent cells [25,28](Figure 2). These junctions are essential for the maintenance of endothelial integrity and regulate the passage of ions, plasma proteins and circulating leukocytes between endothelial cells [29,30]. The junctional structures are built up by trans-membranal protein complexes known as tight junctions (TJ) and adherent junctions (AJ). While TJ are established by homophilic interactions between members of the claudin family such as claudin-3 and -5, occludins and junctional adhesion molecules (JAM), AJ mostly consist of vascular endothelial cadherin (VE-cadherin) [28,29]. The stability and integrity of both junctional structures are controlled by actin cytoskeletal dynamics. This function depends on the anchorage of the cortical actin filaments to AJ and TJ through different adapter proteins such as members of the zonula occludens family (ZO-1, -2 and -3) in the case of the TJ and members of the catenin family (α-, β- and γ-catenin) in the case of the AJ. Actin cytoskeletal remodeling is coordinated by actin-binding proteins (ABP) and small GTPases of the Rho family such as Rac1, RhoA and Cdc42, and Rap1 (Figure 2). Under basal conditions, Rac1 and Cdc42 stabilize AJ by controlling the formation of cortical actin filaments [31]. For example, p21-activated kinase (PAK), a specific Rac1 effector, activates LIM kinase (LIMK) which phosphorylates cofilin-1 to inhibit its actin-

depolymerization activity [32,33]. Additionality, the ABP cortactin is recruited to the periphery to support the assembly and reorganization of cortical actin filaments and to control the RhoA/ROCK pathway to prevent stress fiber formation [34–37]. On the other hand, during inflammation, endothelial cells activate RhoA and its effector Rho kinase (ROCK) that phosphorylates myosin light chain (MLC) and inhibits myosin light chain (MLC) phosphatase leading to the formation of contractile actomyosin stress fibers that exert puling forces on junctions leading to the destabilization of cell contacts [38,39].

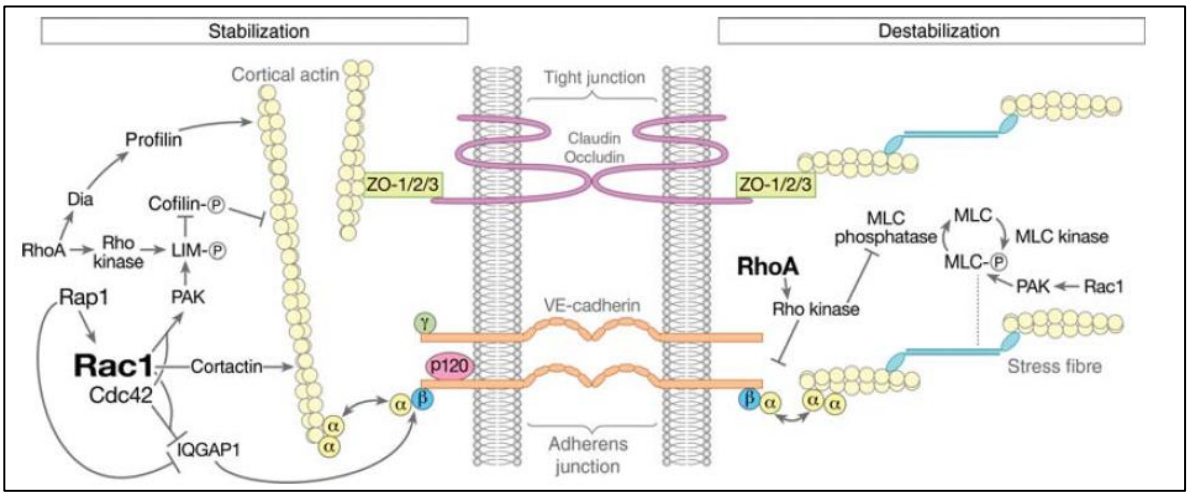

Figure 2. **Mechanisms of endothelial junction regulation**. Endothelial cell junctions are connected to cortical actin filaments via adaptor proteins such as ZOs and catenins. Actin filament rearrangements control the stabilization/destabilization of the endothelial barrier and are regulated by actin-binding proteins (ABP) and small GTPases. While Rap1, Rac1 and Cdc42 stabilize the cell contacts via formation of cortical actin filaments, the RhoA pathway destabilizes the endothelial contacts via formation of contractile actomyosin stress fibers [39].

During extravasation, the luminal neutrophil-endothelial cell interactions induce a cascade of signaling pathways in endothelial cells that control the junctional remodeling to support neutrophil TEM, which will be described below [25].

1.3.1.1. Neutrophil trans-endothelial migration

Neutrophil recruitment begins with the local activation of the venular wall in response to inflammatory mediators such as IL-1β, TNF-α, IL-17. Endothelial

activation includes an increase in the expression of adhesion molecules, mostly intercellular adhesion molecule-1, -2 (ICAM-1, ICAM-2) and vascular cell adhesion molecule-1 (VCAM-1), E-selectin and P-selectin, and the presentation of chemokines on the surface of endothelial cells (Figure 3). Low-affinity interactions of endothelial P- and E-selectins with PSGL1 (P-selectin glycoprotein ligand-1) and ESL1 (E-selectin ligand-1) on neutrophils mediate neutrophil capturing onto the stimulated endothelium followed by neutrophil rolling along the endothelial apical surface with the blood flow. During rolling, selectin engagement and chemokine recognition by the neutrophil chemokine receptors such as CXCR2 induce activation of the β2-integrins LFA-1 (αLβ2 o CD11a/CD18) and Mac-1 (αMβ2 o CD11b/CD18) and subsequent high-affinity binding to their ligands ICAM-1 and ICAM-2, thus allowing for firm adhesion. Then, neutrophils reach the site of diapedesis by rearranging their actin cytoskeleton, spreading on the endothelial surface, and intravascular crawling through Mac-1/ICAM-1 interactions [23,27,40–42]. Prior to crossing the endothelial wall, a docking structure enriched in ICAM-1, VCAM-1 and actin is formed by which endothelial cells surround the adherent neutrophil to guide it to the preferred site of transmigration. The formation of this structure is regulated by recruitment of adaptor molecules such as cortactin, filamin B, α-actinin, ezrin, radixin, moesin (ERM) proteins and signaling molecules such as Rac1 and RhoG to coordinate the actin remodeling and adhesion molecule clustering at the apical endothelial surface for protrusion formation [43–47]. These docking structures have been reported to support diapedesis via both the paracellular and transcellular routes [48,49].

Breaching the endothelial cell barrier can occur through junctions between adjacent cells (paracellular route) or through the endothelial cell body (transcellular route). The paracellular route is preferred by around 80-90% of neutrophils [50] and this requires the transient and reversible breaching of intercellular junctions. Neutrophil-endothelial interactions during diapedesis occur in a sequential manner beginning with the formation of ICAM-1 clustering at the site of exit followed by sequential interactions of neutrophils with the endothelial cell junction proteins JAM-A, PECAM-1 and CD99, respectively [6,27,42]. In addition, the transient loss of VE-cadherin from the endothelial junctions is required for paracellular neutrophil TEM. Internalization of VE-cadherin is

induced by clustering of selectins and ICAM-1 that trigger the recruitment and activation of RhoA and Rac1 that control the activity of Src kinase, PYK2 and vascular-endothelial protein tyrosine phosphatase (VE-PTP) at the endothelial cell contacts. This drives the dissociation of VE-PTP from the VE-cadherin/catenin complex and Tyr phosphorylation of VE-cadherin and β-catenin by Src kinase and PYK2. Together with increased actomyosin contractility, this leads to the transient loss of VE-cadherin via endosomal internalization and the crossing of neutrophils through the endothelial cell contacts [25,51–53].

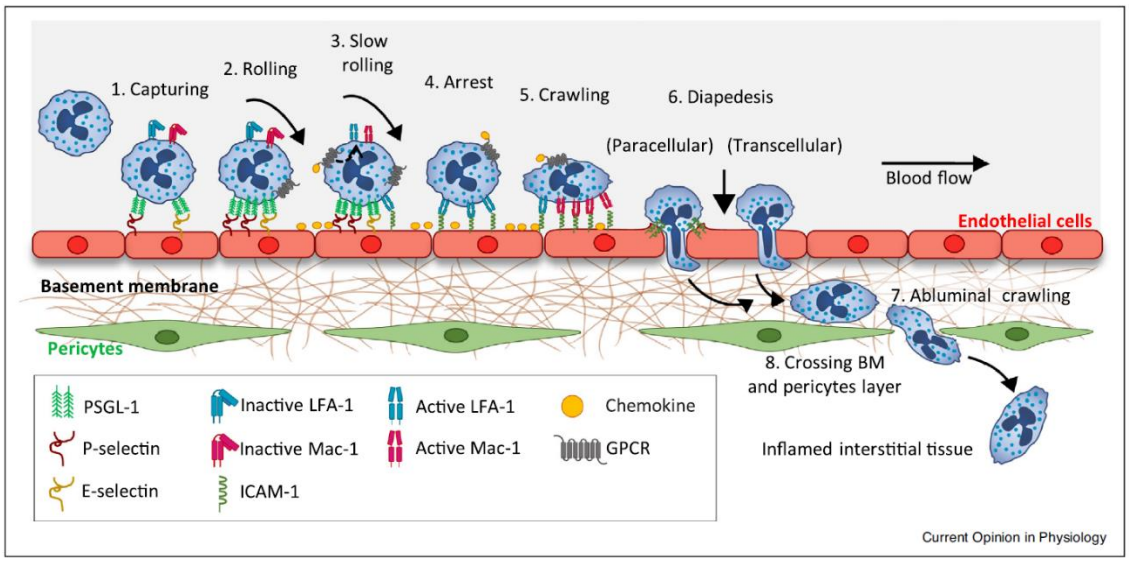

Figure 3. The neutrophil trans-endothelial migration cascade. The sequential stages of neutrophil recruitment are shown, as well as the main adhesion molecules that mediate each step of the cascade in the venular wall and the neutrophil [54].

1.3.2. Crossing the basement membrane

The vascular basement membrane (BM) is the noncellular component of the venular wall, which is composed of tightly packed networks of different extracellular matrix proteins including laminins (mostly laminin 8 and laminin 10) and collagen type IV that are interconnected by other glycoproteins such as nidogen-2 and perlecan[55,56]. These matrix proteins are secreted by both endothelial cells and pericytes and other non-venular cells such as epithelial cells[56–58]. The BM plays a role in maintaining the integrity of the endothelial and pericyte layers and provide a structural support for migrating leukocytes.

Once the endothelial cell layer has been breached, neutrophils must pass through the basement membrane prior to crossing the pericyte layer in order to enter the interstitial space [55]. However, due to the difficulties of generating a venular BM *in vitro*, little is known about how neutrophils breach it. Using confocal intravital microscopy (IVM) of mouse cremaster venules, it has been shown that neutrophils exhibit motility in the abluminal side of the endothelial cell layer and that they extend membrane protrusions to find permissive sites in the BM with low presence of matrix proteins to traverse it [59,60]. Interestingly, these low-expression regions (LER) are closely associated with the gaps formed between adjacent pericytes; and neutrophils can exploit these sites as preferred “gates” to exit from the venular wall (Figure 4) [59,61].

Currently, the role of BM remodeling in leukocyte extravasation is still debated. Some studies have shown that transmigrating neutrophils can promote the proteolytic cleavage of laminin through secretion of neutrophil elastase resulting in an increase of these LER [59,61]. On the other hand, endothelial cell-derived enzymes have also been associated with digestion of vascular laminins 8 and 10 in response to proinflammatory stimuli [56,62,63]. However, the mechanisms through which endothelial cells or neutrophils regulate remodeling of the BM remain poorly understood.

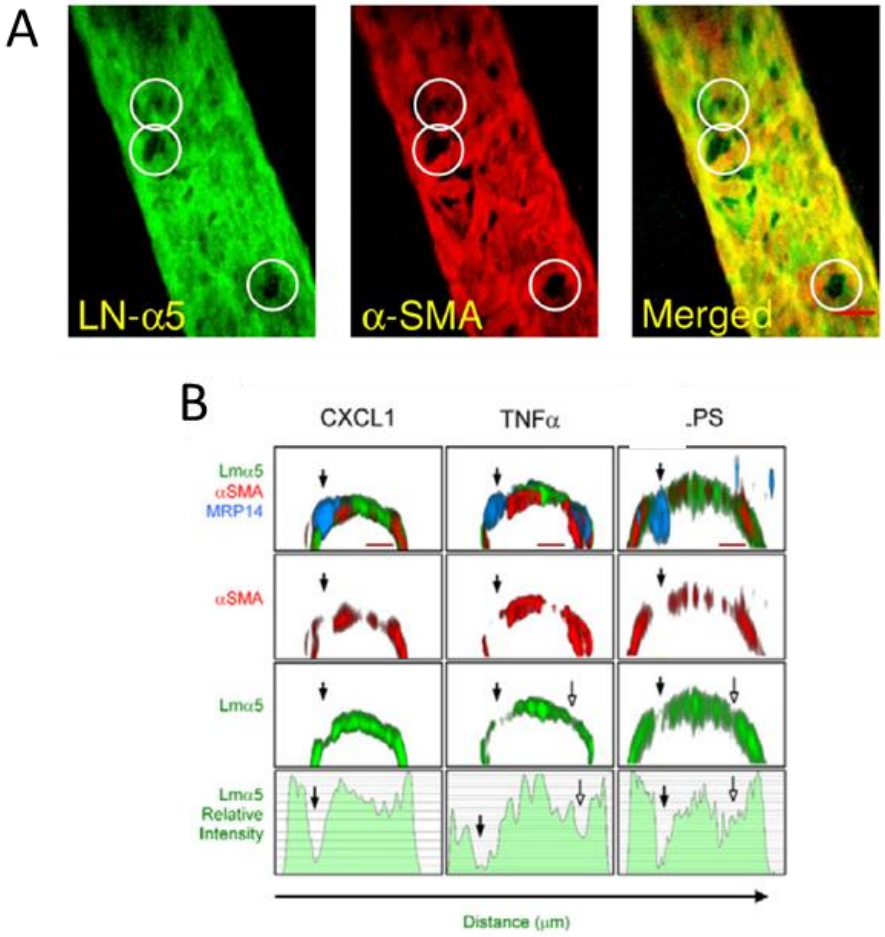

Figure 4. Neutrophils exploit low-expression regions (LER) of the BM as exit points from the venular wall. (A) Representative three-dimensional images of unstimulated cremasteric venules immunostained for laminin-α5 chain (detecting laminin 10 in green) and α-SMA (as pericyte marker in red). White rings indicate LER sites of laminin 10 highly associated with the localization of pericytes gaps. (B) Representative latitudinal cross sections of cremasteric venules locally stimulated with CXCL1, TNFα or LPS and immunostained for laminin-5 (green), α-SMA (red) and MRP-14 (blue, as neutrophil marker). The confocal images show that neutrophils transmigrate preferentially through LERs (filled arrows), whereas the regions with higher relative intensity of laminin-5 do not support neutrophil migration (open arrows) [59,60].

1.3.3. Trans-pericyte migration (TPM): pericytes support neutrophil subendothelial crawling

Pericytes are spatially isolated contractile cells with long and stellate morphology reaching a length of 150 to 200 µm. They are embedded within the venular basement membrane [64]. In contrast to endothelial cells, which form a confluent polarized monolayer, pericytes are loosely distributed surrounding the abluminal surface of capillaries and post-capillary venules, and get in contact through their many long protrusions, thus forming a discontinuous layer with gaps between adjacent pericytes [60,65]. These contractile cells play an important role in maintaining the integrity of the venular wall and contribute to the generation of the vascular basement membrane (BM) [58,65,66]. Venular pericytes are characterized by the expression of platelet-derived growth factor receptor-β (PDGFRβ), the proteoglycan NG2, which is a co-receptor for PDGF, and α-smooth muscle actin (α-SMA) that forms many actin fibers to provide contractility [67]. Interestingly, several studies have shown the ability of pericytes to change their shape in response to inflammatory mediators [67,68]. By confocal microscopy of cremaster and ear skin venules, a recent study showed that pericytes respond to TNF-α and IL-1β stimulation by increased elongation and gaps formation [68]. Apparently, this response supports neutrophil migration through the pericyte layer. However, the nature and signaling pathways that regulate these pericyte shape changes remain unclear. It is thought that actin, ABP, and GTPase-mediated cytoskeleton rearrangements trigger this response.

Although considerable advances have been made in understanding the mechanisms that mediate neutrophil adhesion and diapedesis through the endothelium [69–71], little is known about the subsequent events that mediate neutrophil migration through the pericyte layer. Several studies using confocal microscopy of several tissues have shown that neutrophils breach the pericyte layer through gaps between adjacent pericytes that form in response to several inflammatory stimuli such as IL-1β, TNF, CXCL1, LPS and ischaemia–reperfusion injury [59–61]. In addition, a more detailed *in vivo* study using confocal intravital microscopy of TNF-stimulated cremasteric venules revealed that neutrophils crawl along the pericyte protrusions to find the gaps prior to transmigrating across the pericyte layer (Figure 5). The mechanistic analysis

showed that pericytes up-regulate the expression of ICAM-1 in response to TNF stimulation. In addition, locally administrated blocking antibodies against ICAM-1, Mac-1 or LFA-1 resulted in impaired neutrophil crawling on pericytes leading to overall inhibition of neutrophil extravasation [68]. The authors concluded that this "*abluminal crawling*" is actively supported by interactions of pericyte ICAM-1 and its neutrophil ligands Mac-1 and LFA-1 [68]. Finally, neutrophils emigrate into the extravascular tissue, where leukocytes move through the interstitium towards sites of inflammation.

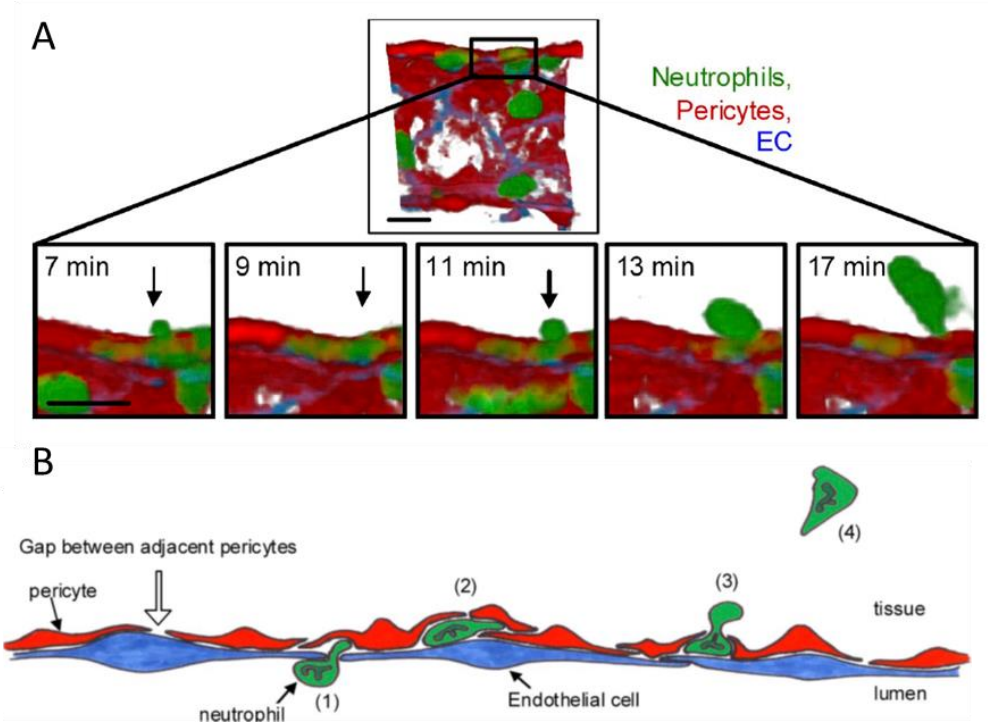

Figure 5. Neutrophils crawl along the pericyte processes prior to breaching the pericyte layer. (A) 3D images acquired by confocal intravital microscopy of TNF-stimulated cremasteric venules from α-SMA-RFPcherry; Lys-EGFP-ki mice exhibiting labeled pericytes with RFP (red) and neutrophils with EGFP (green), with endothelial cells being labeled using a PECAM-1-AF647 antibody (blue). 3D real-time imaging shows neutrophil crawling through the pericyte sheath at the indicated time points. The arrows represent a neutrophil exhibiting a protrusion toward the extravascular space prior to exiting completely from the pericyte layer. (B) Schematic representation of neutrophil migration through the pericyte layer. (1) the neutrophil breaches the endothelial cell layer (TEM), (2) the neutrophil crawls between the endothelial and pericyte layer (abluminal crawling), (3) the neutrophil migrates across gaps between adjacent pericytes, and (4) reaches the interstitial tissue [72].

1.4. Cortactin, a versatile actin-binding protein

Neutrophil extravasation requires all components of the venular wall to be highly dynamic and sensitive to different stimuli to control expression of adhesion molecules, signaling pathways, secretion of ECM proteins, junctional remodeling, specialized membrane protrusion formation and cellular contraction. Actin cytoskeletal remodeling plays a central role in controlling many of these cellular functions. This actin dynamic includes the assembly, disassembly, branching or severing of actin filament networks, processes which are all coordinated by signaling molecules such as small GTPases and ABP [45,73].

Cortactin is an ABP initially identified as an 80/85 kDa substrate of Src kinase that targets actin filaments at the cell cortex, hence its name [74,75]. Expression of cortactin has been reported to be almost ubiquitous except for some hematopoietic cells that instead express the cortactin homologue HS1 [76]. The protein structure is organized into functionally distinct domains through which cortactin interacts with a plethora of actin regulatory proteins (Figure 6) [75,77,78]. It has an N-terminal acidic (NTA) domain that interacts directly with the Arp2/3 complex, followed by 6.5 tandem repeats mediating actin filament binding [79,80]. Next is a helical region of unknown functions, followed by the central region containing a proline-rich domain abundant in serine, threonine, and tyrosine residues, which are targets for phosphorylations that mediate binding to proteins containing SH2/SH3 domains [37]. Finally, the C-terminal end contains an SH3 domain through which cortactin can interact with many other actin-regulatory proteins or scaffolding proteins [37,75,81]. Given this network of interactions, cortactin regulates many cell functions including cell migration, cell-cell adhesion, extracellular matrix deposition and vesicular trafficking by controlling actin cytoskeletal rearrangements, activation of GTPases and scaffolding other proteins into functional entities [75].

Cortactin interacts with the Arp2/3 complex that mediates the assembly of new branched actin filaments on existing filaments [82,83]. However, cortactin itself is a weak activator of the Arp2/3 complex, and instead, can recruit and increase the activity of other nucleation-promoting factors (NPF) such as N-WASP and stabilize the newly branched actin filaments by preventing debranching or depolymerization [84,85]. Cortactin localizes to many actin-rich structures regulated

by the Arp2/3 complex such as lamellipodia, membrane ruffles, sites of endocytosis, exocytosis, vesicular trafficking, cell-cell adhesions, podosomes, invadopodia, and also transmigratory cups [86,87].

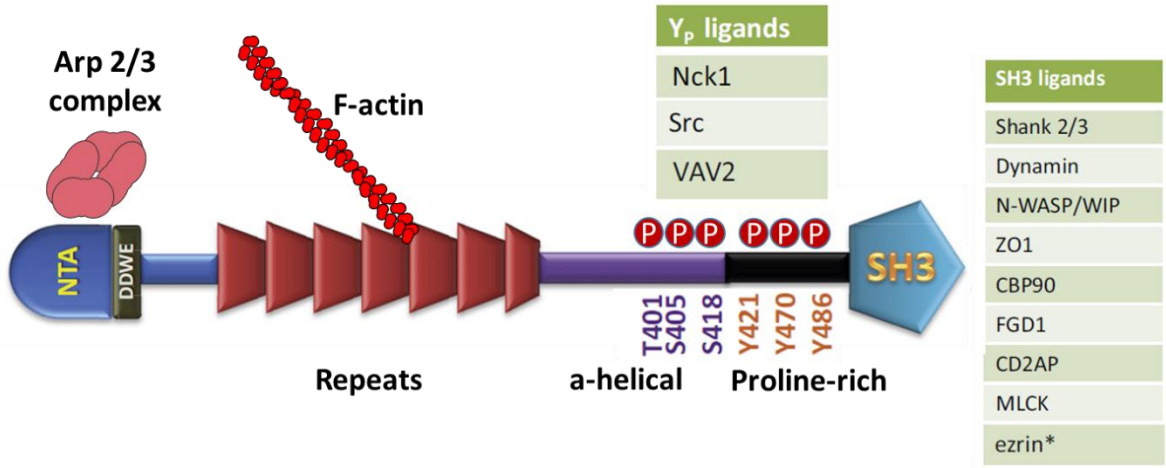

Figure 6. Cortactin domain structure. Schematic representation of the domain organization, post-translational modifications and principal molecules that interact with cortactin. Modified from Schnoor et al., 2018 [75].

1.4.1. Cortactin regulates endothelial barrier functions and neutrophil TEM

Several studies have described essential roles of cortactin in endothelial cells. For example, it is involved in regulating vascular permeability and neutrophil trans-endothelial migration by controlling actin cytoskeletal remodeling, clustering of adhesion molecules and activation of GTPases. Recent *in vivo* and *in vitro* works showed that cortactin-deficient mice have increased basal permeability due to reduced basal Rap1 activity and increased ROCK-mediated MLC phosphorylation leading to increased contractile stress fiber formation and junction destabilization that collectively contribute to hyperpermeability [34,48] (Figure 7). Interestingly, transmigrating neutrophil cannot exploit the loose endothelial cell contacts, and, instead, cortactin deficiency reduced neutrophil transmigration *in vivo* and *in vitro* (Figure 8) [48]. This reduced neutrophil extravasation was due to defective adhesive interactions of neutrophils with endothelial apical ICAM-1 leading to increased rolling velocity and reduced

neutrophil adhesion. In this context, cortactin was required for RhoG-mediated ICAM-1 clustering into ring-like structures surrounding adherent neutrophils [48,88–90]. Moreover, phosphorylated cortactin bound directly to E-selectin to support E-selectin clustering and subsequent leukocyte adhesion onto the endothelial apical surface [91]. Taken together these findings demonstrate that cortactin is required for proper neutrophil-endothelial interactions through regulation of apical adhesion molecule clustering.

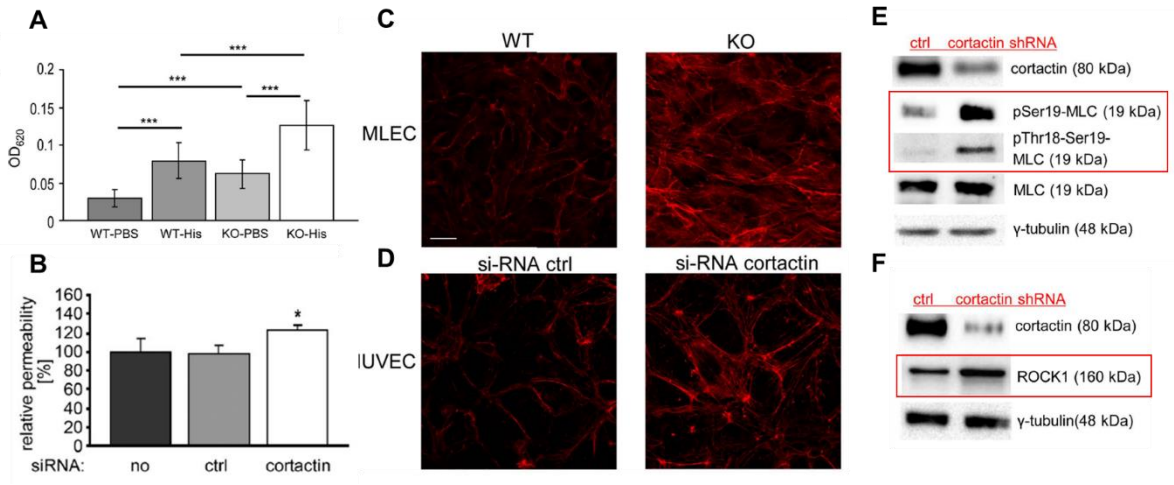

Figure 7. Cortactin regulates the endothelial barrier function. (A) In vivo permeability measured by leakage of Evans blue after intradermal administration of histamine or PBS alone in the back skin show increased permeability in cortactin-deficient (KO) mice than wild type (WT) mice. (B) In vitro paracellular permeability for 250-kDa FITC-dextran through WT or KD HUVEC monolayer on transwell filters (0.4 μm pore size) show higher permeability in cortactin KD HUVEC[48]. (C-D) Higher permeability was associated with increased formation of actin filaments in both cortactin deficient MLEC and HUVEC. (C-D) Representative confocal images of actin filaments (phalloidin) from wild type (WT) or cortactin-deficient (KO) murine lung endothelial cells (MLEC) (C), and WT (si-RNA control) or cortactin-depleted (si-RNA cortactin) human umbilical vein endothelial cells (HUVEC) (E-F). Immunoblots of effector molecules involved in actin stress fiber formaction, MLC, pMLC and ROCK1 showing increased levels in cortactin KD HUVEC vs WT HUVEC [34].

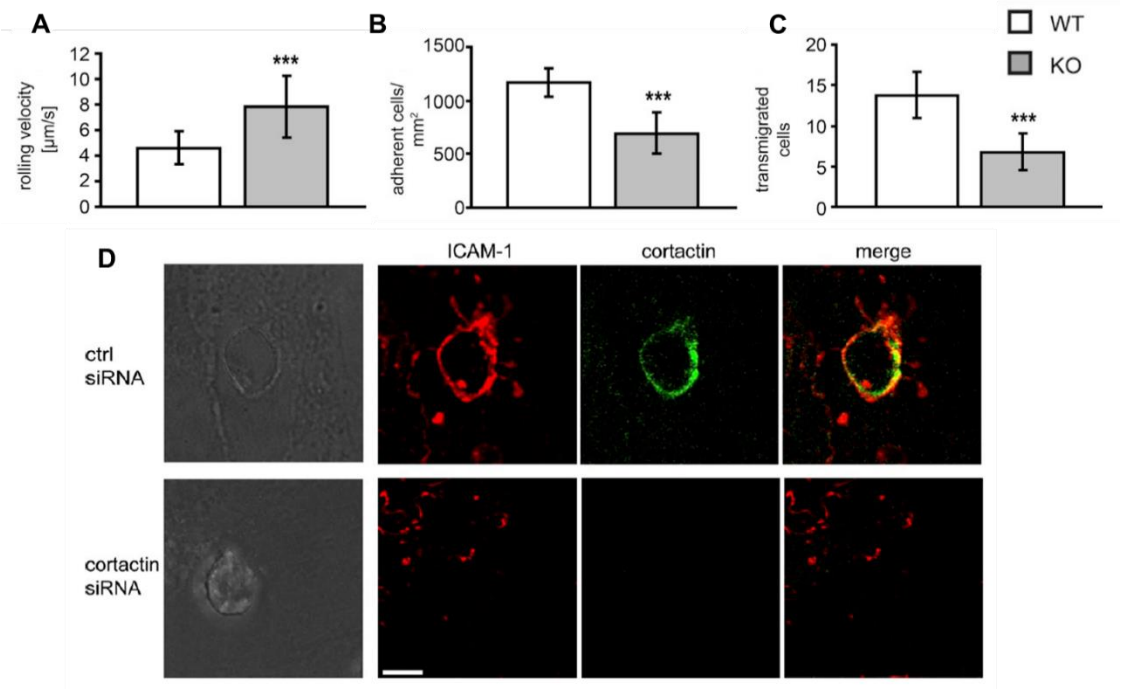

Figure 8. Cortactin modulates neutrophil extravasation. (A-C) Quantitative analysis of intravital microscopy of mouse cremaster muscle stimulated with TNFα from WT and KO mice. (A) Reduced rolling velocity and (B) neutrophil adhesion was observed in the absence of cortactin. (C) These defects lead to a reduced number of neutrophil transmigration. (D) Confocal immunofluoresence of ICAM-1 (red) and cortactin (green) in co-cultures of WT and cortactin KD HUVEC monolayers with human neutrophils for 20 minutes. Showing defective formation of ICAM-1 clusters in the absence of cortactin [48].

1.5. Neutrophil effector responses in the inflamed interstitium

1.5.1. Neutrophil granules

Neutrophils are armed with a large array of proteases and antimicrobial peptides that are stored in their cytoplasmic granules, and their release represents a crucial microbicidal weapon during their effector response at the inflamed extravascular tissue. Based on their content, granules that are formed from the *trans*-Golgi network are classified into three types: azurophil granules (primary) containing mainly myeloperoxidase (MPO), neutrophil elastase, cathepsin G, proteinase 3 and inducible nitric oxide synthase (iNOS); specific granules (secondary) containing lactoferrin and lysozyme; and gelatinase granules (tertiary) containing mainly gelatinase and metalloproteinases (Figure 9) [10,92,93]. Neutrophils also contain secretory granules (also called secretory vesicles, SV)

that are formed by endocytosis and rapidly mobilize proteins that need to be transported to the cell surface such as CD11b/CD18 [17,94]. The granule subsets can be released by exocytosis in response to inflammatory stimuli or by recognizing pathogens [95]. Secretory vesicles are quickly transported to the cell membrane in response to weak stimulation such as chemoattractans (IL-8, fMLP) or signaling by selectin during endothelial interaction, to increase the presence of more adhesion molecules and chemokine receptors needed for neutrophil extravasation such as Mac-1 and CXCR2 [11]. The secretion of gelatinase and specific granules occur in response to stronger stimuli such as the pro-inflammatory cytokines TNFα or pathogens-derived molecules, whereas the release of primary granules depends on priming [11].

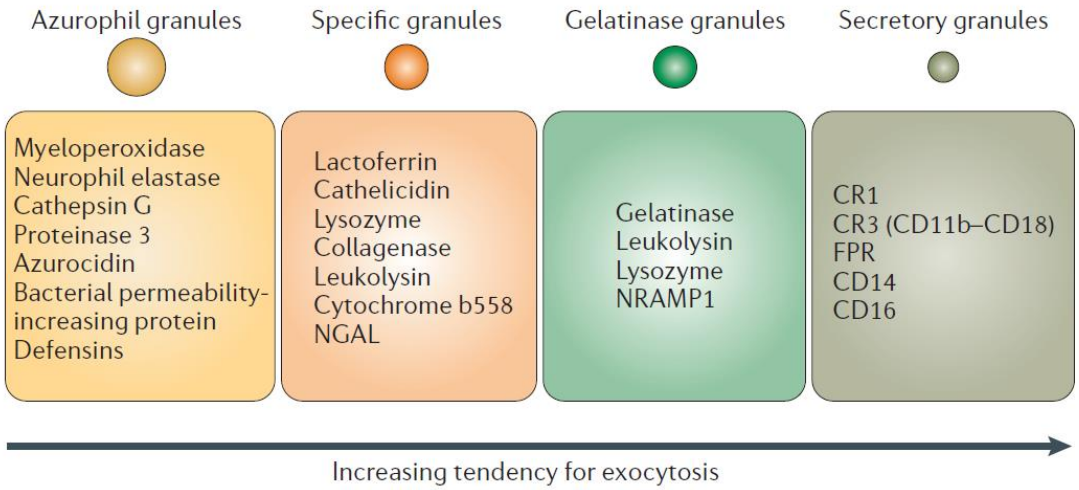

Figure 9. Neutrophil granular content. Diagram illustrating the variety of proteins stored in the different class of neutrophil granules. Granules are organized according to their ability to be released after stimulation. Secretory granules are the most easily exocytosed, followed by gelatinase granules and specific granules, while azurophil granules only undergo limited secretion. CR, complement receptor; FPR, formyl peptide receptor; NGAL, neutrophil gelatinase-associated lipocalin; NRAMP1, natural-resistance-associated macrophage protein 1 [95].

1.5.2. Neutrophil serine proteases (NSP): definition and proteolytic properties

Neutrophil serine proteases (NSP) are a class of 29-35 kDa proteolytic enzymes with anti-microbial activity. They represent the most toxic cargoes from the neutrophil granules and some of the most-abundant neutrophil proteins [96–98]. NSP include neutrophil elastase (NE), proteinase 3 (PR3), cathepsin G (CG) and

the recently identified neutrophil serine protease 4 (NSP4) [99], which all belong to the superfamily of chymotrypsin-like serine proteases. NSP are characterized by the presence of a conserved serine residue in their catalytic triad His^{57}-Asp^{102}-Ser^{195}, which is formed by protein folding during maturation. Each protease possesses different affinity for cleaving peptide bonds between amino acids at the P1 and P1' cleave site (Figure 10). For example, PR3, and NE preferentially cleave the hydrophobic amino acids V, C, A, and the polar T amino acid, whereas CG more commonly hydrolyses hydrophobic (L and M) or aromatic (F and Y) amino acids. By contrast, NSP4 hydrolyses more efficiently after arginine residues [97,99,100]. All NSP share the neutral amino acids G, A and S at the P1' position. Due to the similarity of the amino acid sequence of the substrate located in the catalytic pocket, all NSP share many substrates [97,100–102]. NSP exhibit their optimal enzymatic activity at neutral pH [95,97].

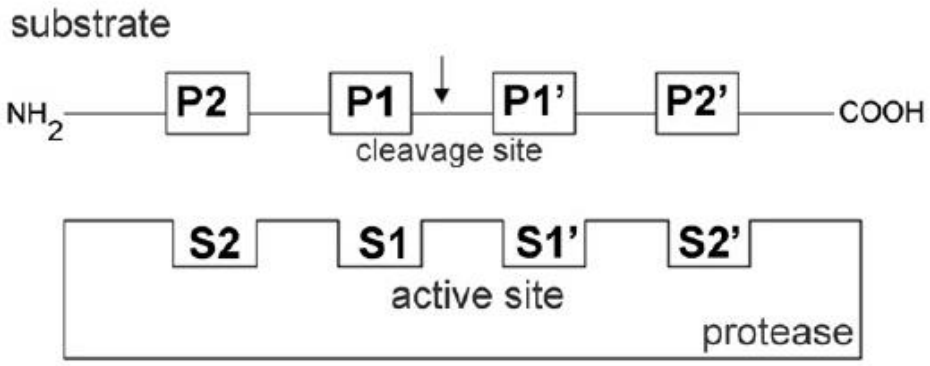

Figure 10. Substrate cleavage by proteases. Diagram of the Schechter and Berger (1967) nomenclature that illustrates the interactions between a protease and a substrate. S subsites on the protease bind to the amino acid residues in the substrate sequence (P residues) and cleave peptide bonds between P1 and P1' (cleavage site). P1-P2 indicate the amino acid positions from the cleavage site toward the N-terminus of the substrate, whereas P1'-P2' represents the amino acid positions toward the C-terminus [103].

1.5.3. Production and maturation of NSP

The biosynthesis of NSP is regulated at both transcriptional levels and post-translational level before storing in azurophil granules. NSP are produced during the maturation of neutrophils in the bone marrow [10] . The transcription of NSP

occurs mainly during the pro-myelocytic stage, during which the transcription factors PU.1, cMyc, C/EBPα up-regulate the levels of mRNA expression of the NSP that later are down-regulated at subsequent maturation stages [10,95,104–106] (Figure 11).

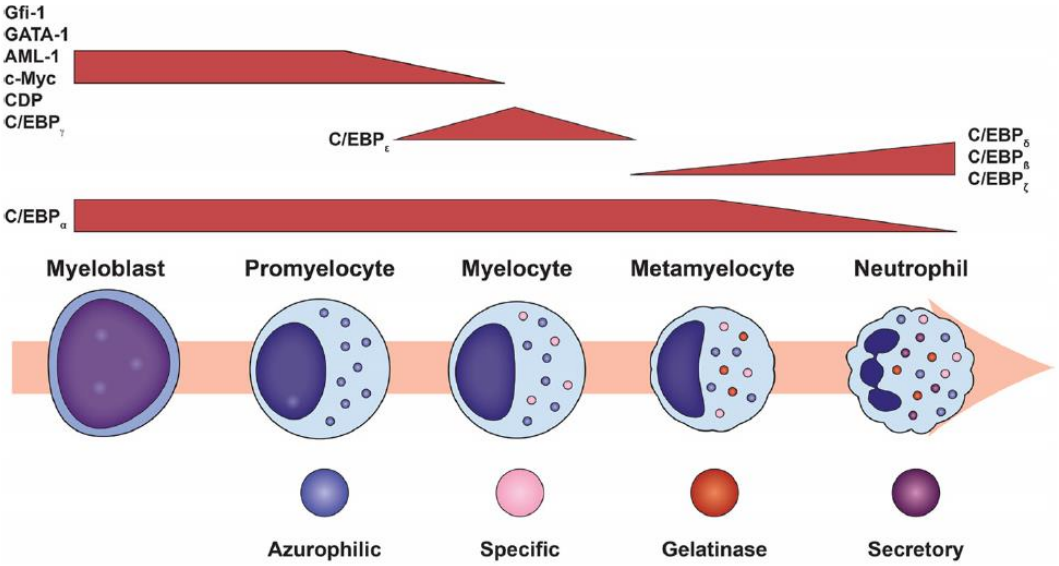

Figure 11. Biosynthesis of granules during granulopoiesis. The maturation of neutrophils within the bone marrow is characterized by the sequential formation of the granules and secretory vesicles. The transcription factors that are expressed during the different stages of maturation of neutrophils are indicated in red. This array of transcription factors determines the up or down regulation of genes from the different set of granules. Genes involved in the production of azurophilic granules are up-regulated by PU.1 and cMyc during the promyelocyte stage and are later down-regulated in the myelocyte stage. Specific granules are produced during this stage by C/EBPε, whereas gelatinase granules are expressed in metamyelocyte stage [107].

After translation, NSP are subjected to post-translational modifications to become fully active before they are stored in azurophilic granules. NSP are synthesized as inactive zymogens containing a signal peptide and prodipeptide in their N-terminus, and a propeptide in their C-terminus (Figure 12). These pre-pro-NSP undergo proteolytic processing in 3 sequential steps to form the active enzyme. After synthesis, the N-terminal signal peptide ensures translocation of the pre-pro-NSP along the endoplasmic reticulum (ER) membranes. In the ER lumen, the signal peptide is removed by a signal peptidase leaving the pro-enzyme still inactive. The pro-dipeptide is then cleaved in the Golgi complex by the cysteine

protease dipeptidyl peptidase I (DPPI), also known as cathepsin C (CTSC) [95,97,108]. This N-terminal processing results in correct folding of the catalytic site (His[57]-Asp[102]-Ser[195]) thus forming the active protease. Then, the C-terminal extension is cleaved by a yet unknown mechanism, although this does not seem to be required for full enzymatic activity [103,109]. The mature NSP are finally stored in their enzymatically active form within the azurophil granules by yet unknown mechanisms for rapid release in response to specific stimuli [95,97].

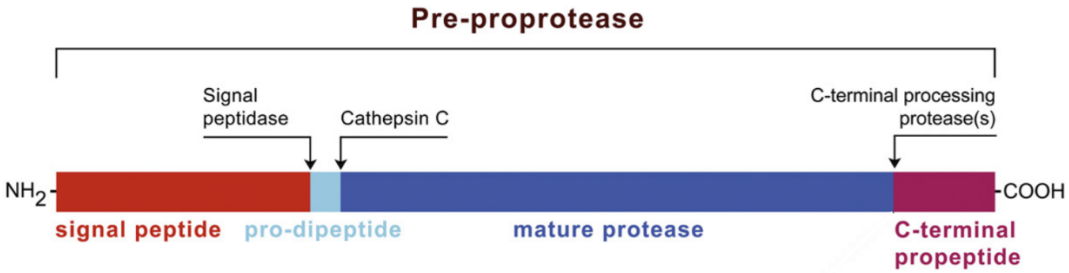

Figure 12. Structure of the neutrophil serine pre-proteases. NSP are traduced as a pro-protease consisting of an N-terminal signal peptide followed by a prodipeptide, the protease sequence and a C-terminal extension. The mature protease is generated by proteolytic cleaving of the signal peptide by a signal peptidase, then cathepsin C cleaves the pro-dipeptide, whereas the processing of C-terminal propeptide is carried out by an unknown protease [110].

Although NSPs are mainly stored in azurophilic granules, they have been also found in other compartments. For example, proteomic analyses have identified NE, PR3 and CG in neutrophil extracellular vesicles (EVs) and EV-like structures released by neutrophils during intravascular rolling [111–116]. PR3, but not NE and CG, has been also detected in secretory vesicles, specific granules, and on the cell surface of resting neutrophils [117]. Interestingly, NETs also contain NE, PR3 and CG [113,118].

1.5.4. Exocytosis of azurophilic granules

As mentioned above, exocytosis of azurophilic granules is limited due to the high toxicity of their cargoes. For example, after stimulation with fMLP or extravasating neutrophils, approximately 100% of SV, 38% of gelatinase granules, 22 % of specific granules, and only 7% of azurophil granules are released [119]. It is well

established that neutrophils require a pre-stimulus to release azurophil granules, however, the exact mechanism controlling their exocytosis is not yet well defined. Pre-exposure to cytokines (TNF-α), chemoattractants (fMLP or IL-8), LPS or adhesion molecules can lead to the mobilization of azurophilic granules to the cell membrane [103]. Priming with TNF-α followed by fMLP stimulation of human neutrophils in vitro, but not TNF-α or fMLP alone, induced the release the azurophilic granules [120,121]. Other studies have shown that the release of azurophilic granules is enhanced by LPS pre-treatment followed by fMLP stimulation [122,123]. Pre-treatment with TNF-α, and IL-1β also increases the release of azurophilic granules in response to IL-8 [124,125]. Importantly, the cross-linking of L-selectin followed by IL-8 stimulation induces the exocytosis of azurophilic granules, but not cross-linking of L-selectin, Mac-1 (CD11b/CD18), CD45 or CD31 alone [126,127]. Interestingly, adhesion to fibrinogen and fibronectin does not induce the release of azurophilic granules [128,129]. Adherent neutrophils to platelet monolayers or P-selectin are able to release elastase in response to IL-8, fMLP and C5a, but lower secretion of elastase was detected when adhesion to platelets was blocked with monoclonal anti-CD18 or anti-CD11b antibodies indicating that neutrophil-platelets interactions via β-2 integrins induce the secretion of NE[130]. On the other hand, only 26% secretion of elastase from human neutrophils was observed after CD18 crosslinking [131]. Likewise, CD18 ligation to soluble ICAM-1 was shown to induce secretion of elastase in human neutrophils [132]. Numerous in vivo studies have shown the release of NSP during neutrophil extravasation [133–135]. Of note, neutrophils release NE intravascularly in close proximity to endothelial junctions in response to LTB4 stimulation [133].

Intracytoplasmic azurophil granules are secreted by fusion with the plasma membrane in a tightly controlled mechanism of exocytosis. Their translocation to the plasma membrane depends on cytoskeleton remodeling, microtubule assembly and the small Rab GTPases soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein receptors (SNAREs) and vesicle-associated membrane proteins (VAMPs) [103,136]. Although the exact mechanisms regulating the selective mobilization of azurophil granules is not fully understood, it has been suggested that the differential combination of SNARE complexes present in azurophil granules determines the selective exocytosis. The rise of intracellular

Ca^{2+} concentration is the first step to facilitate the interaction between the vesicle-SNARE (vSNARE) and the corresponding target membrane (t-SNARE) complexes in the plasma membrane. The release of Ca^{2+} from intracellular stores is achieved by activation of the Src kinases Fgr and Hck, which are triggered by β2-integrin-dependent adhesion or GPCR-ligand interactions. Additionally, this signaling facilitates microtubule polarization and actin remodeling [136,137]. Then, Rab proteins tether vesicles to target membranes, while SNARE proteins mediate their docking and fusion with the plasma membrane. In neutrophils, the tethering of azurophil granules to the plasma membrane is regulated by Rab27 and its effectors JFC1/Slp1 [138], while the selective docking with the plasma membrane is regulated by the interaction of syntaxin-4 with VAMP-1 and VAMP-7 located at azurophil granules [139]. In addition, it is well established that the exocytosis of azurophilic granules requires cortical actin depolymerization. The assembled cortical actin network blocks the fusion of azurophil granules to the plasma membrane [140]. During exocytosis, azurophilic granules fuse in areas of the plasma membrane with lower F-actin density. Supporting this mechanism, the interaction of JFC1 with RhoA induces the inhibition of RhoA, leading to actin depolymerization around the granule, and fusion with plasma membrane [138,140,141].

CD63 has been established as a surface marker for exocytosis of azurophil granules, as increased surface expression of CD63 has been observed after their release [121]. Furthermore, a fraction of PR3, NE and CG can remain bound to the outer cell surface after azurophil granules fused with the plasma membrane during exocytosis, with the possibility to exert proteolytic activity on substrates in close proximity to the neutrophil surface [97,103].

1.5.5. Neutrophil serine proteases exert several inflammatory effects

NSP are key players of the non-oxidative mechanisms of neutrophils to destroy intracellular and extracellular pathogens. Once neutrophils arrive at the inflamed interstitial tissue, the main aim of granule exocytosis and NSP release is extracellular bacterial killing [11]. NE degrades the plasma membrane of gram-negative bacteria by cleaving the outer membrane protein A (OmpA) and

virulence factors [97,142]. Additionally, extracellular PR3 processes cathelicidin (CAP-18) to its active LL-37 form, which exerts a broad antimicrobial activity against Gram-negative and Gram-positive bacteria [143]. NE, GC and PR3 can also act against pathogens without exerting enzymatic degradation. For example, they can bind to negatively charged bacterial membranes via their positive surface charges to induce membrane depolarization and disruption [103].The importance of NSP in host defense is further highlighted by the higher susceptibility of NE-deficient and CG-deficient mice to Gram-negative and -positive bacteria, as well as to fungal infections leading to lower survival [97,144–147].

Besides their functions in pathogen degradation, NSP are also recognized as multifunctional enzymes with an important role in the regulation of non-infectious inflammatory responses. Extracellular NSP can regulate the activation or inactivation of chemokines, cytokines, as well as specific receptors by direct proteolytic processing [103]. The pro-inflammatory cytokines TNF-α and IL-1β are synthesized as inactive pro-forms bound to the cell surface and require processing by TNF-converting enzyme and caspase-1, respectively, to generate the active form. PR3 can cleave IL-1β into its active form, while both PR3 and HNE can generate active TNF-α [103,148,149]. The generation of the bioactive forms of IL-18 [150], IL-32α [151], TGF-β [152] and IL-33 [153] also involve cleaving by NSPs [97]. The major neutrophil chemokine IL-8 (also known as CXCL8) is secreted by neutrophils, monocytes, endothelial cells, and fibroblast. The full-length-IL-8 is susceptible to processing by PR3 generating a 10-fold more active truncated IL-8 with higher chemotactic activity [103,154]. Likewise, the chemokines CXCL5 (also known as ENA78) and CCL15 (also known as MIP1δ) are cleaved by cathepsin G, leading to enhanced chemotactic activity of neutrophils and monocytes, respectively [155,156]. By contrast, NE and GC are involved in the inactivation of SDF-1α (also known as CXCL12) and its receptor CXCR4 by processing both molecules, whereas all three NSPs have shown to cleave CCL3 (also known as MIP1α) leading to reduced chemotactic activity of lymphocytes [157–160].

Some specific cellular receptors have shown susceptibility to proteolysis by NSP, resulting in the activation or inactivation of the receptor. NE, PR3 and CG can cleave the extracellular domains of protease-activated receptors (PARs). While the processing of PAR-4 in platelets by CG induces its activation leading to

platelet aggregation [161], the processing of PAR-1 and PAR3 by all three NSPs results in the inactivation of the receptor [162–164]. Likewise, NE cleaves and activates PAR-2 in nociceptive neurons to induce inflammation and pain [165]. In addition, the C5a receptor can be inactivated in myeloid cells by CG, NE and PR3 [166]. NE has also been implicated in the inactivation of surface CXCR1 by direct proteolytic cleavage, which ultimately results in impaired host defense [167,168]. All NSP can also degrade extracellular matrix (ECM) proteins such as elastin, collagen IV, laminin, vitronectin, and fibronectin [169–171]. The proteolytic cleavage of these ECM proteins has been associated with neutrophil migration through the endothelial cell barrier, the perivascular basement membrane, and the extravascular interstitial tissue, which will be further described below.

Due to this wide-ranging substrates and functions, NSP have been involved in numerous pathological scenarios, in which NSP are excessively released and lead to tissue damage, vascular dysfunction and systemic inflammation. This often occurs under conditions when the inflammatory response is not controlled well, such as chronic inflammatory lung diseases, autoimmune vasculitis, arthritis, cardiovascular diseases and sepsis (37). For example, excessive secretion of NSP can result in destruction of lung extracellular matrix [103]. Furthermore, membrane bound-PR3 in circulating resting neutrophils is the major autoantigen for the generation of anti-neutrophil cytoplasmic autoantibodies (ANCA) that are involved in development of Wegener granulomatosis, a systemic autoimmune vasculitis. In patients with this condition, ANCA bind to surface-PR3 to induce the activation of neutrophils in the circulation leading to necrotizing vasculitis of small vessels [97,172]. On the other hand, congenital deficiency of NSP is also associated with human diseases. Whereas mutations in ELA2/ELANE the gene encoding NE are a cause of human neutropenia, mutation in the gene encoding cathepsin C or DPPI (CTSC) affect the production of all three NSPs and is the cause of Papillon- Lefèvre Syndrome, a hereditary skin disease with higher risk to recurrent infections [95,103].

1.5.6. Endogenous inhibitors of NSP

To reduce the side-effects of NSP, three families of endogenous protein inhibitors exist that antagonize their enzymatic activities: serpins (serine protease

inhibitors), chelonianins, and macroglobulins [95]. The serpin family is mainly represented by two high-molecular weight inhibitors, α1-Protease inhibitor (α1-PI) and α1-antichymotrypsin (ACT). They are secreted by hepatocytes into the plasma and diffuse into the extravascular space during inflammation when vascular permeability is increased. α1-PI, also known as α1-antitrypsin (α1-AT or AAT), binds and inhibits NE, PR3 and CG, whereas ACT targets mainly CG. Neutrophils and macrophages also express the cytoplasmic SerpinB1 (also known as MNEI – monocyte neutrophil elastase inhibitor), which inhibits mostly NE and CG, although it also targets PR3 with lower affinity. In addition, the serpin PI9 is expressed by endothelial cells, but inhibits NE less efficiently than all the above [103,110]. Serpins act as bait-substrates that bind to the proteases by presenting their reactive site. The protease recognizes that site as potential substrate and cleaves the reactive center loop of the serpin. This reaction leads to the distortion of the catalytic site in the protease forming a complex, in which the serpin remains trapped inside the NSP leading to an irreversible inactivation of the protease.

The canonical chelonianin family of inhibitors are of small-molecular weight and includes the secretory leukocyte proteinase inhibitor (SLPI), elafin and its precursor, trappin-2. They are locally secreted by goblet cells, bronchial and alveolar epithelial cells, neutrophils and macrophages into mucosal surfaces and skin [95,173]. SLPI inhibits NE and CG, whereas Elafin also known as “elastase specific inhibitor”, is a potent inhibitor of NE and PR3 [103,110]. Similar to the inhibitory mechanism of serpin, chelonianin inhibitors are recognized as pseudo-substrates by NSP through an exposed binding loop that binds to the active site of the enzyme. The enzyme-inhibitor complex prevents the protease to recognize and cleave a real substrate. However, by contrast to serpin, the inhibition of chelonianin inhibitors is reversible [103,173].

The broad-spectrum protease inhibitor α2-macroglobulin (α2M) in the blood inhibits all classes of proteases, including NSP. α2M rarely diffuses into extravascular tissue due to its large molecular mass, and therefore its function is mainly exerted in the circulation. Its inhibition mechanism requires the protease to recognize and cleave the bait region by its active site. This reaction induces a conformational change in α2M, forming a tetrameric cage around the protease

that sterically shields its active site. The resulting trapped protease is then unable to cleave large substrates but can still cleave small substrates [103,174].

Although these inhibitors appear to be excellent mechanisms to regulate the side effects of NSP, neutrophils use different mechanisms to avoid the activity of these endogenous inhibitors. When neutrophils release NSP in very narrow compartments, for example, when interacting with other cells, junctional compartments, or extracellular matrices, high molecular weight inhibitors such as AAT or ACT hardly reach into those tight spaces, leaving the enzymatic activity of NSP intact [97]. Furthermore, surface-associated NSP, have been shown to be more resistant to inhibition by high-molecular weight inhibitors compared to soluble NSP [117,175,176]. Likewise, the inhibitory activity of AAT is lower for NE when it is bound to tissular proteins such as elastin [177]. Neutrophils also possess enzymatic mechanisms to control the activity of these inhibitors. Neutrophil proteases such as MMP-8 can inactivate ACT, whereas MMP-9, MMP-7 and cathepsin L can inactivate AAT [103]. Similarly, the cysteine proteases cathepsin B, L and S can inactivate SLPI [103].

The importance of the inhibitory function of serpins is demonstrated in individuals with genetic variants of AAT harboring single amino acid substitutions that lead to reduced levels or total deficiency of AAT [178]. Patients with AAT deficiency show higher risk to develop pulmonary emphysema, thrombosis, angioedema, and cancer mainly due to uncontrolled enzymatic activity of NSP [103,178]. Furthermore, chronic obstructive lung disease, ANCA-positive vasculitis and hepatic dysfunction are often associated with AAT deficiency. AAT replacement therapy consisting in intravenous infusion of serum-purified AAT have been effectively applied to these patients during the last 30 years [179].

1.5.7. Neutrophil-derived extracellular vesicles modulate inflammation

Besides granules and secretory vesicles, neutrophils also release extracellular vesicles (EVs), which exert a diversity of regulatory functions depending on their cargoes, the stimuli, and the targeted cell. EVs are a highly heterogenous group of membrane derived vesicles bordered by phospholipid bilayers that are secreted spontaneously, upon stimulation or during apoptosis [180]. EVs carry

biologically active molecules such as proteins, lipids, and nucleic acids. Based on their size and biogenesis, EVs can be classified as exosomes, microvesicles, and apoptotic bodies (Figure 13) [181]. Exosomes (30-100 nm) are produced by the intracellular endosomal pathway and are stored into multivesicular bodies (also called multivesicular endosomes-MVE). They are released by fusion of MVE with the plasma membrane. Some endocytic markers are present in neutrophil exosomes such as CD63, CD9 and CD81 tetraspanins. By contrast, the microvesicles or ectosomes (100-1000 nm), are formed by outward budding of the plasma membrane, followed by their fission and release into the extracellular space. Apoptotic bodies (> 1000 nm) are also released by budding from apoptotic cells [180,181].

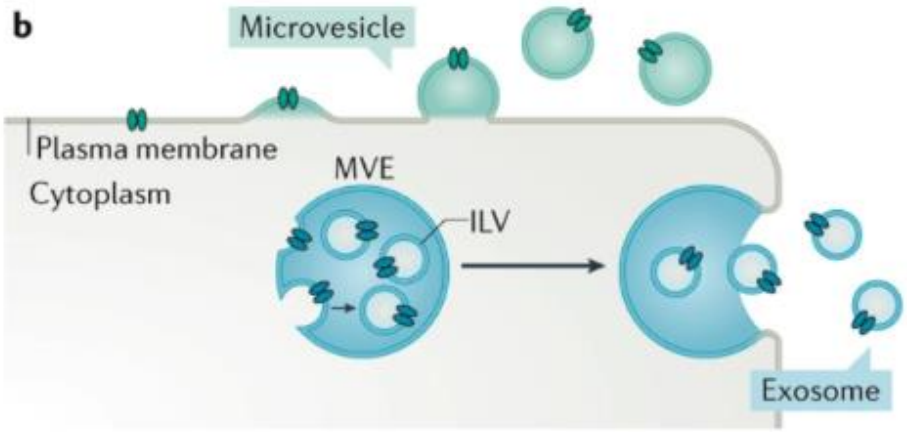

Figure 13. Biogenesis of extracellular vesicles. Illustration of the mechanisms of generation of microvesicles and exososomes. Microvesicles are produced directly by budding off from the plasma membrane, while exosomes are formed as intraluminal vesicles (ILV) stored within the lumen of multivesicular endosomes (MVE) that are released by fusion of the MVE with the plasma membrane [181]**.**

Once released, EVs are captured by target cells via specific receptors that recognize the ligands from the source cell to establish an intercellular communication. However, the exact mechanisms that mediate the interaction of EVs and the transfer of their cargoes into the recipient cells are not fully understood. EVs undergo docking at the target plasma membrane, followed by the activation of surface receptor and downstream signaling to induce its internalization via different mechanisms (Figure 14). EVs can also be directly

captured by their fusion with the plasma membrane of target cells to deliver their content. Inside the recipient cells, the contents of the EVs can exert functional responses and induce phenotypic changes.

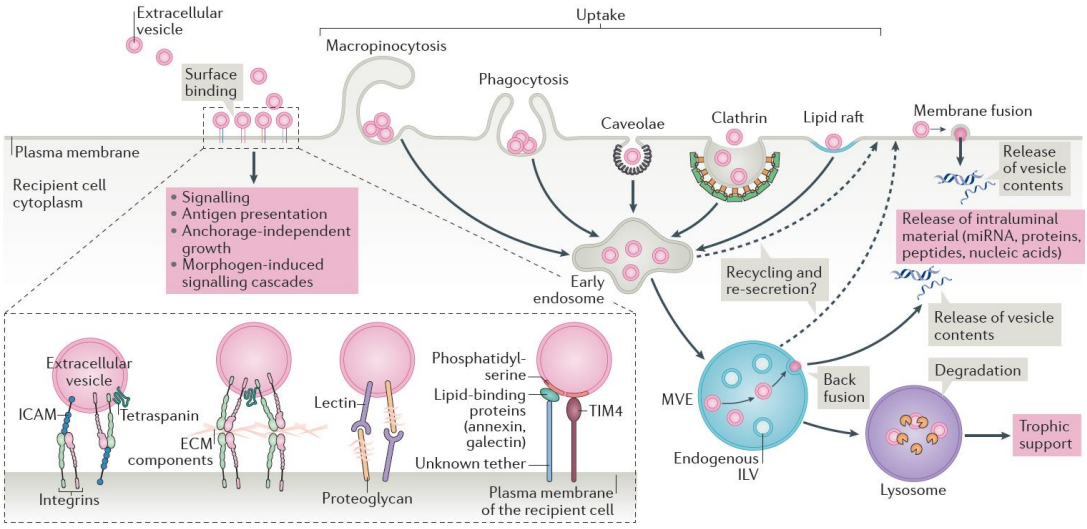

Figure 14. Uptake and intracellular fate of extracellular vesicles in the recipient cell. EVs bind to the target plasma membrane via the interactions of molecules expressed at the surface of EVs and recipient cells such as tetraspanins, integrins, adhesion molecules, lipids, lectins, proteoglycans and extracellular matrix (ECM) components, among others. The array of these ligands and receptors likely determines the target cell specificity. Bound at the cell surface, EVs can remain at the plasma membrane, or can be internalized by macropinocytosis, phagocytosis, clathrin-mediated endocytosis, caveolae, lipids rafts or membrane fusion. Internalized EVs can follow the endocytic pathway until reaching the lysosomes for degradation. In some cases, EV can fuse with the membrane of the MVE and release their contents into the cytoplasm [181].

The size of neutrophil EVs usually vary from 100 to 700 nm and they contain typical neutrophil surface markers such as CD11b, CD62L, CD16, CD18, and granule-associated markers such as CD63, CD66b and MPO, and they can be labelled with annexin V due to PS exposure [180,182]. Several proteomic studies analyzing neutrophil EV content detected mainly proteins from the cytoskeleton, granules (MPO, lactoferrin, neutrophil elastase, matrix metallopeptidase 9 and

proteinase 3), and mitochondria [180,182]. Neutrophil EVs are released mainly during immune responses, however, neutrophils also release EVs spontaneously in circulation. Increased secretion of EVs from neutrophils have been reported upon a variety of conditions and in response to different stimuli. Pathogens and their products (LPS, fMLP or zymosan), opsonized particles and TNF-α are the strongest stimuli that induce the release of EVs compared to spontaneous EV production [180,182]. EVs can also be secreted in response to the neutrophil chemokine IL-8, complement fragments C5a, IFN-γ, anti-neutrophil cytoplasmic antibody (ANCA), PAF and GM-CSF [180,182]. Accordingly, the plasma of patients with sepsis, community-acquired pneumonia, and ANCA-associated vasculitis contain high levels of neutrophil EVs [111,183–186]. In addition to serum, neutrophil EVs have also been detected in bronchoalveolar fluids of patients with pneumonia and sepsis, in blisters of wound, and abdominal fluids of septic patients (6, 22, 28).

Neutrophil-derived EVs are important regulators of local and systemic inflammation. Based on their cargoes, neutrophil EVs may transmit pro-inflammatory or anti-inflammatory signals to the target cell and induce functional changes. Target cells are mainly other neutrophils, macrophages, endothelial cells, and epithelial cells. In circulation, neutrophil EVs bind to RBC through CR1 or integrin interactions and induce their aggregation. Neutrophil EVs can also bind to endothelial cells via CD18/CD11b that respond by increasing the expression of the pro-inflammatory cytokines IL-6, IL-8, and ROS [185,187]. On the other hand, macrophages respond to neutrophil EVs by decreasing the production of the pro-inflammatory cytokines TNF-a, IL-6, IL-8 and IL-10, whereas the anti-inflammatory cytokine TGF-β is increased in monocytes, macrophages and NK cells exposed to neutrophil EVs [182].

Although the main role is intercellular communication, neutrophil EVs also exhibit direct intrinsic immune functions without capture by a target cell, with the most described function of neutrophil EVs being anti-microbial activity. EVs can directly bind to bacteria via CR1, induce its aggregation and inhibit their growth [111,114,188]. In addition, neutrophil EVs can generate ROS and LTB4 in response to fMLP [116]. Moreover, neutrophil EVs can deliver granule proteins such as MPO to intestinal epithelial cells leading to impaired wound healing [189], and NE-bound exosomes

can degrade lung extracellular matrix leading to pulmonary emphysema in mice [190].

Neutrophils that adhere to endothelial cells also release EVs during extravasation, although the mechanisms are still not well understood. The elongated uropod of migrating neutrophils can detach from the neutrophil and generate small vesicles that remain attached to the endothelium thus forming migrasomes. This type of EVs has been observed in the lung vasculature of mice intratracheally infected with influenza virus-, and in peripheral vessels of mice infected with *Leishmania major* and *Candida albicans*. As they contain mainly chemokines such as IL-16, CCL2, CCL5, CCL6, CCL12, CCL22, CCL27, CXCL1, and CXCL12, these migrasomes act as cues to guide the migration of other immune cells to the site of inflammation. However, their role in inducing functional changes in the recipient cells including endothelial cells has not been investigated [191,192]. Another recent work observed by intravital microscopy elongated neutrophil derived structures (ENDS) during rolling of neutrophils in an endotoxemia model. ENDS were formed by detachment of tethers from rolling neutrophils on E and P-selectin, and contain S100A8-S100A9, cathepsin G, and proteinase 3, among others. ENDS are also positive for PS, CD66b and CD16, but negative for the EVs markers CD9, CD63 and CD81. These particles were detected 10-100fold higher in blood plasma of septic patients compared to healthy individuals. On the other hand, none of these studies investigated the functional relevance of these structures for the process of neutrophil extravasation itself [112].

1.5.8. Neutrophil extravasation is modulated by NSP

NSP can be released into the intravascular and sub-endothelial space throughout the neutrophil extravasation process. Therefore, NSPs have been often associated with neutrophil infiltration in different pathological conditions, however, results on the role of NSPs in modulating adhesion and diapedesis have been controversial. Numerous studies have demonstrated that the inhibition or deficiency of NSPs reduces neutrophil infiltration to different organs under distinct conditions. NE and CG inhibition results in diminished neutrophil infiltration into

I/R-injured skeletal muscle [193]. Furthermore, NE-deficient mice have shown reduced neutrophil recruitment, into myocardium, kidney, cremaster muscle, and mesentery after I/R injury, as well as reduced adhesion and transmigration in the cremaster muscle after intrascrotal zymosan administration [134,194]. By contrast, NE deficiency or inhibition did neither affect the adhesion nor extravasation of neutrophils when cremasteric venules were stimulated with the cytokines TNF-α or IL-1β [134]. The NE and CG inhibitor Eglin C efficiently impairs the adhesion and trans-endothelial migration of neutrophils through mesentery post-capillary venules stimulated with PAF [195]. Furthermore, the inhibition of NE and CG by the recombinant serpin LEX032 leads to reduced infiltration of neutrophils into the peritoneal cavity in a rat model of glycogen-peritonitis [196]. Importantly, in vitro studies have supported these in vivo observations. NE has been observed at the front of migrating neutrophils through HUVEC monolayers in response to PAF. In this model, the secretion of NE was required to efficiently adhere and migrate through endothelial cells [197,198]. Likewise, in vitro inhibition of PR3 using Elafin or AEBSF results in diminished neutrophil transmigration across HUVEC monolayer [199]. Collectively, these studies demonstrated an important role of NSPs in the regulation of neutrophil trans-endothelial migration. However, some studies have found that neutrophils do not require NSPs to extravasate. For example, the number of recruited neutrophils into the lung and peritoneum was not affected in NE-deficient mice after LPS or *Pseudomonas aeruginosa* challenge, and no effect was observed in the migration of NE-deficient neutrophils through Matrigel [200]. Similarly, CG-deficient and WT mice have shown similar numbers of neutrophils infiltrated into the peritoneum of *S. aureus*-[201].

NSPs have also been implicated in neutrophil migration through the venular basement membrane (BM). At the sub-endothelial space, secreted NE by extravasating neutrophils could remodel the venular BM to form the LERs described in section 1.3.2. These spaces formed by NE were used by neutrophils to completely emigrate from the sub-endothelial compartment [59,60,194]. Deficiency or inhibition of NE resulted in the accumulation of neutrophils at the sub-endothelial space in I/R-injured or IL-1β-stimulated mouse cremaster muscle, leading to lower egress of neutrophils from the venular wall [59,194].

Collectively, these studies suggest a stimulus-specific role for NSPs as regulators of neutrophil extravasation. In addition, it is highly probable that the deficiency of either NE or CG or PR3 is compensated by another, which could be the reason for the contradictory results. Therefore, more investigation is required to understand the mechanisms through which NSPs modulate neutrophil trafficking.

1.6. Neutrophils regulate venular integrity

1.6.1. Neutrophil EVs as intercellular communication with endothelial cells

During inflammation, extravasating neutrophils can alter the integrity of the microvasculature. For example, the adhesion and rolling of neutrophils on the endothelial lumen via ICAM-1 has been shown to increase microvascular permeability in vivo [202]. Likewise, EVs and secreted mediators by neutrophils represent mechanisms for communication with the venular endothelium. Several reports have demonstrated that neutrophil-derived EVs induce a pro-inflammatory phenotype in endothelial cells [116,180]. HUVEC monolayers exposed to neutrophil EVs released by stimulation with fMLP or ANCA, responded by increasing the expression of ICAM-1, secretion of IL-8 and IL-6, and enhancing the production of endothelial ROS [185,187]. In addition, human brain microvascular endothelial cells internalized neutrophil EVs and significantly increased permeability after exposure to fMLP-stimulated neutrophil-EVs [203]. By contrast, using multiphoton intravital microscopy of mouse cremaster muscles, it has been observed that extravasating neutrophils deposited microparticles at the sub-endothelial space during the late stages of trans-migration. These microparticles were essential to maintain endothelial barrier integrity in vivo as the inhibition of their formation resulted in increased vascular permeability [191,204].

Although it is well stablished that endothelial cells respond to neutrophil EVs by acquiring a pro-inflammatory phenotype, the mechanisms through which neutrophil EVs induce those functional changes in endothelial cells remain unclear. Given the well-known immunomodulatory role of NSPs, it is likely that NSPs may be delivered into endothelial cells through EVs and induce pro-inflammatory effects from within, a hypothesis we want to analyze here.

1.6.2. The endothelial response is modulated by neutrophil serine proteases

How the NSP released during extravasation affects endothelial barrier stability has been poorly investigated. Currently, NSP are often associated with increased vascular permeability. VE-cadherin is a target for enzymatic degradation by extracellular CG and NE in vitro leading to increased permeability [205,206]. However, there is no in vivo evidence of direct cleaving of VE-cadherin by NSP. A recent study observed by confocal intravital microscopy of mouse cremaster muscles that neutrophils released NE in close proximity to endothelial junctions in response to LTB4 stimulation. Released NE at those compartments could cleave junctional JAM-C leading to enhanced reverse transmigration [133]. Furthermore, increased albumin leakage into the lung, heart and liver after LTB4 stimulation, was reduced to basal levels in NE-deficient mice [133]. Reduced vascular permeability has also been observed in I/R-injured cremaster muscles from NE-deficient mice, and this response was associated with retention of neutrophils at the sub-endothelial space. Inhibition of NSP by Eglin C led to reduced vascular permeability in canine skeletal muscle subjected to I/R [193].

Apart from the enzymatic processing of junctional molecules, NE can bind to endothelial PAR-2 and trigger intracellular signaling leading to increased permeability. This intracellular response is associated with increased contractile actin filaments and decreased VE-cadherin expression [207]. By contrast, the activation of PAR-2 by PR3 is associated with increased endothelial barrier stability [208].

Recent evidence suggests that NSP can be transferred into endothelial cells, exert proteolytic activity on intracellular substrates, and thereby affect cellular functions. An in vitro study showed that endothelial cells can internalize NSP from the supernatant of fMLP- or ANCA-stimulated neutrophils. Internalized NSP maintained their enzymatic activity and were able to process intracellular endothelial cytoskeletal proteins such as actin, tubulin, vinculin, and vimentin [100]. Ultimately, internalization of NSPs was associated with altered actin cytoskeletal architecture and enhanced permeability, which was abrogated using the serine proteases inhibitor PMSF [100]. In addition, NF-κB and p21/Waf have been also

processed by NSP leading to endothelial apoptosis [209,210]. Internalization of NSPs have also been observed in breast and lung cancer cells [97], but the mechanisms of internalization remain elusive. However, it is likely that NSP are delivered into endothelial cells via EVs or specific endothelial receptors.

II. JUSTIFICATION

Neutrophil recruitment into inflamed tissues is a fundamental event during inflammatory responses. However, uncontrolled neutrophil extravasation can also contribute to severe tissue damage and the pathogenesis of inflammatory disorders. Anti-inflammatory therapies targeting neutrophil mediated-tissue damage often result in immunosuppression and susceptibility to secondary infections. Therefore, a better understanding of the mechanisms that regulate neutrophil trafficking may lead to improved therapeutic strategies that balance the protective vs destructive roles of neutrophils. It is now well established that neutrophil extravasation requires interactions with endothelial cells that need to be tightly controlled to avoid vascular damage while neutrophils pass through the venular wall. Although considerable progress has been made in understanding the adhesive and migratory events that govern neutrophil crossing through the endothelium, the mechanisms regulating endothelial changes in response to neutrophil adhesion remain unclear and need further exploration. Endothelial cortactin is known to control neutrophil-endothelial interactions, and endothelial barrier stability by regulating actin cytoskeletal dynamics. Furthermore, the delivery of neutrophil EVs and NSPs into endothelial cells has recently been shown to drive a pro-inflammatory endothelial response by different mechanisms including cytoskeletal rearrangements. Nevertheless, whether the internalization of NSPs by endothelial cells represents a mechanism triggering neutrophil extravasation is unknown and requires further investigation. Thus, we aimed to analyze whether endothelial cortactin is a target of internalized NSPs to facilitate neutrophil passage through the vascular endothelium.

III. HYPOTHESIS

The expression, localization, and function of cortactin in endothelial cells are regulated by neutrophil serine proteases during inflammation to reorganize the actin cytoskeleton and junctional architecture thereby supporting neutrophil extravasation.

IV. GENERAL AIM

To investigate whether NSP-mediated cortactin processing facilitates neutrophil trans-endothelial migration.

V. PARTICULAR AIMS

1. To characterize the expression and localization of cortactin in endothelial cells under different inflammatory conditions.
2. To analyze the role of neutrophil serine proteases in the regulation of actin remodeling, endothelial integrity, and cortactin dynamics.
3. To analyze the function of cortactin and NSP in the regulation of neutrophil extravasation.

VI. MATERIAL AND METHODS

Table 1. Buffers, solutions, and cell culture media

1X HBSS	8000 mg/L NaCl 400 mg/L KCl 40 mg/L Na_2HPO_4 60 mg/L KH_2PO_4 350 mg/L $NaHCO_3$ 1000 mg/L D-glucose pH 7.4
1X PBS	138 mM NaCl 3 mM KCl 8.1 mM Na_2HPO_4 1.5 mM KH_2PO_4
1X Triton X-100 lysis buffer	2% Triton X-100 150mM NaCl 20mM Tris-HCl pH 8.0 1mM CaCl2 15 µg/ml Leupeptin 1mM PMSF 20 µg/ml Aprotinin
1X SDS lysis buffer	SDS 1% 25 mM HEPES 2 mM EDTA 25 mM NaF, pH 7.6 2x cOmplete, Roche 2x PhosSTOP, Roche 2mM Na3VO4
5X Laemmli buffer	0.1875 M Tris-HCl pH 6.8 45% glycerol 2.5% SDS 1.78 M β-mercaptoethanol 0.00125% bromophenol blue

Blocking Buffer	5 % skim-milk or 5% BSA (SIGMA) TBS-Tween 0.01%
SDS-PAGE buffer	25 mM Tris 192 mM glycine 0.1% SDS pH 8.3
TBS	150 mM NaCl 10 mM Tris base pH 8.0
TBS-Tween 0.1%	100 ml 10x TBS 0.1% Tween 20
Transfer buffer	20% methanol 25 mM Tris 192 mM glycine 0.1% SDS
Hypotonic solution, pH=7.4	0.2% NaCl 1% BSA 20 mM Hepes
Hypertonic solution, pH=7.4	1.6% NaCl 1% BSA 20 mM HEPES
RPMI-1640 Medium	Sigma #R4130 10% FBS
Endothelial Cell Medium (ECM)	ScienCell™ Research Laboratories #1001 5 µg/mL ECGS 10% FBS 1% penicillin/streptomycin

Table 2. Reagents and antibodies

Antibodies

Anti-cortactin (clone 289H10)	Donated by Dr. Klemens Rottner, TU Braunschweig, Germany
Anti-CD31 (Clone 390)	eBioscience, #16-0311-38
Anti-cathepsin G	Invitrogen, #PA5-99402
Anti-proteinase 3	Invitrogen, #PA5-85928
Anti-neutrophil elastase	Invitrogen, #PA5-115648
Anti-MRP-14	US Biological #376767
Goat Alexa Fluor 488 anti-rabbit IgG (H+L)	Invitrogen, #A-11008
Goat Alexa Fluor 647 anti-rabbit IgG (H+L)	Invitrogen, #A-21244
Goat Alexa Fluor 488 anti-mouse IgG (H+L)	Invitrogen, #A-11001
Goat Alexa Fluor 568 anti-mouse IgG (H+L)	Invitrogen, #A-11004
Goat Alexa Fluor 647 anti-mouse IgG (H+L)	Invitrogen, #A-21235
Goat anti-mouse IgG-HRP	Santa Cruz, #sc-2005
Goat anti-rabbit IgG-HRP	Santa Cruz, #sc-2357
Anti-GAPDH (0411)	Santa Cruz, #sc-47724
Anti-γ Tubulin Monoclonal Antibody (4D11)	ThermoFisher Scientific #MA1-850
Ultra-LEAF™ Purified anti-mouse Ly-6G/Ly-6C (Gr-1) Antibody (clone: RB6-8C6)	Biolegend, # 108435
Ultra-LEAF™ Purified Mouse IgG2b, κ Isotype Ctrl Antibody	Biolegend, # 401216
APC/Cyanine7 anti-mouse Ly-6G Antibody	Biolegend, # 127624
Pacific Blue anti-human CD45 Antibody	Biolegend, # 304022
TruStain FcX™ PLUS (anti-mouse CD16/32) Antibody	Biolegend #156604
Reagents	
Murine TNFα	PeproTech, #315-01A
Murine IL-1β	PeproTech, # 211-11B
Murine KC (CXCL1)	PeproTech, # 250-28
LTB4	Merck, # L0517-25UG
Human recombinant TNFα	PeproTech, #300-01A

Recombinant Human ICAM-1-Fc Chimera (carrier-free)	Biolegend, # 552906
Recombinant Human P-selectin (CD62P) Chimera (carrier-free)	Biolegend, # 797706
Recombinant Human E-selectin (CD62E) Chimera (carrier-free)	Biolegend, #718704
MG-132, Ready Made Solution	Sigma, #M7449-1ML
Calpain Inhibitor I, ALLN	Sigma, #A6185-5MG
Leupeptin	Sigma, #L2884-25MG
PD98059	Sigma, # P215-5MG
Sivelestat sodium salt hydrate (ONO-5046)	Sigma, # S7198-5MG
PMSF Roche	Sigma, # 10837091001
AEBSF	Sigma # A8456-100MG
Alpha-1-antitrypsin (AAT)	Zemaira®, CSL Behring. Supplier: Solutesa S.A. de C.V.
Alexa Fluor 488 Antibody Labeling kit	Invitrogen, #A20181
Alexa Fluor 555 Antibody Labeling kit	Invitrogen, #A20187
Alexa Fluor 647 Antibody Labeling kit	Invitrogen, #A20186
Histopaque-1077 (1.077 g/ml)	SIGMA, #10771
Histopaque-1119 (1.119 g/ml)	SIGMA, #11191
TruStain FcX PLUS (anti-mouse CD16/32) Antibody	Biolegend #156604
Human TruStain FcX (Fc Receptor Blocking Solution)	Biolegend #422301
PFA (Paraformaldehyde)	SIGMA, # 158127-500G
ProLong Gold mountant with Dapi.	Invitrogen, #P36935
AF488 Phalloidin	Invitrogen, #A12379
Rhodamine Phalloidin	Invitrogen, #R415
TrypLE™ Express	Gibco, #12604-013
Trypsin-EDTA 0.25%	Sigma, #T4049-500ML
Saponin	Sigma, #84510-100

SuperSignal West Femto substrates	ThermoFisher, Scientific #34087
30% Acrylamide/bis solution	Bio-Rad, #161-0153
BD Pharm Lyse™	BD Bioscience, #555899
Triton X-100	Sigma, #T9284
Tween® 20	Sigma, #P1379-500ML
ANESKET® (Ketamine)	PiSA, #Q-7833-028
BD Pharm Lyse™	BD Bioscience, #555899
Bovine serum albumin	Sigma, #A2153-100G
Ethylenediaminetetraacetic acid (EDTA)	Sigma, #E9884-500G
Fetal bovine serum	Biowest, #S1810
HEPES	HEPES, #P5455-100GR

6.1. Mice

For the in vivo experiments where cortactin was stained in cremaster muscle, male mice were used at an age of 8-12 weeks and a weight of 20-25 g. For intravital microscopy of cremaster muscle, *LysM-EGFP-ki* male mice were used at the same age and weight as C57BL6 mice. *LysM-EGFP-ki* mice produce green neutrophils that express enhanced GFP (EGFP). They were generated by knocking in the EGFP gene into the murine lysozyme M (LysM) locus (donated by Dr. Sussan Nourshargh, Queen Mary University of London, UK) [211]. Mice were kept in the animal barrier facility at Cinvestav under specific pathogen-free conditions. All experiments have been approved by the Institutional Animal Care and Use Committee (IACUC) of Cinvestav, Mexico. In all experiments, mice were anaesthetized by intraperitoneal (i.p.) injection of ketamine hydrochloride (100 mg/kg) and xylazine (10 mg/kg) and euthanized by anesthesia over-dose followed by cervical dislocation.

6.2. *In vivo* inflammation models

In anesthetized mice, cremaster muscle inflammation was induced by intrascrotal (i.s.) injection of 300 ng TNFα, 50 ng IL-1β, 500ng CXCL1 (PeproTech, USA), 300 ng LTB_4 (R&D Systems, USA) for periods of 2 - 4 h. Control mice were injected i.s. with 400 µl of PBS only. All injected solutions contained 4µL of conjugated anti-PECAM-1 antibody (clone 390) (eBioscience, USA) to label blood vessels.

6.3. Cremaster muscle staining and confocal microscopy

After incubation with inflammatory mediators, cremaster muscles were carefully excised and fixed in 4% paraformaldehyde (PFA) for 45 minutes at 4°C and subsequently permeabilized and blocked in PBS containing 0.5% Triton X-100 and 25% FBS for 4 hours at RT with gentle shaking. Then, whole tissues were transferred into an Eppendorf tube and incubated in 150 µl of PBS containing 10% FBS and primary antibodies [anti-cortactin-AF488 (clone 289H10) and anti-MRP-14-AF647 (clone 2B10, only when C57Bl/6 mice were used instead of LysM-EGFP, to label neutrophils)] overnight at 4°C. Tissues were then washed 3 times with PBS 1X for 15 min at RT. When unlabeled primary antibodies were used, cremaster muscles were then incubated with species-specific fluorescently-labeled secondary antibodies (1:1000) in PBS for 3 hours at RT. Finally, the whole tissues were washed 3 times in PBS and mounted on a glass slide with PBS. If possible, primary antibodies were conjugated to Alexa Fluor 488, 555 or 647 fluorophores using labeling kits (Thermo Fisher Scientific, USA) according to the manufacturer's instructions.

To analyze the presence of neutrophil proteases in the venular endothelium, the whole fixed cremaster muscle was incubated with 1:100 polyclonal rabbit anti-Cathepsin G, anti-Proteinase 3, and anti-Neutrophil elastase antibodies (Invitrogen, USA), followed by incubation with secondary antibody goat-anti-rabbit-AF647 (Invitrogen, USA). Images of post-capillary venules of 20-40 µm diameter were acquired using a confocal laser scanning microscope Leica TCS SP8 or Nikon A1. Analysis of confocal images were performed using the 3D reconstruction software Imaris-Bitplane (Oxford Instruments, UK)

6.4. Neutrophil depletion

To analyze the role of neutrophils in the inflammatory response, mice were subjected to neutrophil depletion by intravenous (i.v.) injection of 25 µg rat anti-Gr-1 antibody (Ly6C/Ly6G clone: RB6-8C6) [212]. Control mice were injected i.v. with 25 µg of IgG2b, κ isotype control antibody. 24 hours later, local inflammation was induced by i.s. injection of TNFα (300 ng), and cremaster muscles were prepared for confocal microscopy as described above. After cremaster muscles were dissected and the mice were euthanized, blood was obtained by cardiac puncture to confirm neutrophil depletion by flow cytometry.

To prepare the blood sample for flow cytometry, erythrocytes were lysed by adding 1 mL of pre-warmed 1x BD lysing buffer (BD bioscience, USA) to 100 µL of blood, according to manufacturer's instructions. Lysis was allowed for 3 minutes at room temperature (RT) protected from the light. 9 mL of sterile 1x PBS was immediately added after 3 minutes of incubation to dilute the lysis solution. Cell suspension was centrifuged at 1500 rpm for 5 minutes. The supernatant was aspirated, and the remained cells were washed twice with 2 mL of 1x PBS containing 3% of fetal bovine serum (FBS). Cell suspensions were then blocked in 100 µL of ice-cold PBS + 3% FBS containing mouse TruStain FcX (1:100) blocking solution (Biolegend, USA) for 30 minutes in ice. Later, cells were washed twice with 250 µL of cold 1x PBS + FBS 3%. Extracellular staining of Ly6G and CD45 was performed by incubating cell suspensions in 100 µL of cold PBS + 3% FBS containing APC/Cy7 anti-Ly6G (1:200) and Pacific blue anti-CD45 (1:200) labelled antibodies for 30 minutes. Subsequently, stained cells were fixed by adding 100 µL of warmed 4% PFA and incubating for 20 minutes at room temperature. Fixed cells were washed twice with cold PBS + 3% FBS and resuspended in 100 µL of the same buffer. Cell suspensions were then acquired in FACS Canto II flow cytometer (Becton Dickinson, USA) and analyzed using FlowJo Treestar V10 software (BD Bioscience, USA).

6.5. AAT augmentation therapy and AEBSF treatment in mice

C57BL6 and *LysM-EGFP-ki* mice were subjected to pharmacological treatment with the irreversible neutrophil serine protease inhibitor (NSP) AEBSF to identify

the role of serine proteases in the degradation of endothelial cortactin during in vivo inflammation. AEBSF (30 mg/kg) was administrated i.p. 1h before inducing intrascrotal inflammation with TNFα for 2 or 4 h. Cremaster muscles were then excised and prepared for immunofluorescence as described above. Alternatively, the specific clinical-grade NSP inhibitor human α1-antitrypsin (AAT, Zemaira®, CSL Behring) was injected i.p. (2 mg per mouse) 2 hours before TNFα intrascrotal injection for 2 and 4 h.

6.6. Isolation of human peripheral blood neutrophils

6 ml of Peripheral blood from healthy donors (with previous informed consent) was collected using BD Vacutainer EDTA K2 tubes (Becton Dickinson, Mexico). Blood leukocyte populations were separated by layering 6 mL of total blood on the top of a Histopaque 1119 and 1077 density gradient (3 ml of solution 1.077 g/ml over 3 ml of 1.119 g/ml solution, SIGMA, USA) and then centrifuged at 700 xg for 30 minutes at room temperature without brakes. Neutrophils were collected from the interface of the Histopaque 1119 and Histopaque 1077 layers and washed twice with 10 ml of ice-cold PBS + 10% FBS. Remaining erythrocytes were lysed by adding 5 ml of cold hypotonic solution for 1 min on ice. 5 ml of hypertonic solution was added immediately thereafter. Isolated neutrophils were then washed twice with cold PBS and resuspended in 5 ml of cold RPMI-1640 (Merck, USA) media supplemented with 10% FBS. The cell suspension was counted in a Neubauer chamber with trypan blue solution (1:1 dilution, 10 µL of cell suspension + 10 µL of trypan blue) to determine the number of live cells that were used in different assays as described below.

6.7. Isolation and culture of Human Umbilical Vein Endothelial Cells (HUVEC)

To establish a primary human umbilical vein endothelial cell culture, discarded umbilical cords were donated from pregnant women during the cesarean (all donors signed an informed consent). Umbilical cords were maintained in sterile PBS during transport to the laboratory for immediate digestion. Subsequently, 1x PBS containing 1% Streptomysin/Penicilin was flushed through the vein using a

sterile cannula inserted into the upper end. Then, one end of the cord was sealed with forceps and 0.25% of trypsin-EDTA (Sigma, USA) was administrated via the canulated upper end until the vein was filled. The other end was sealed, too, and digestion was performed for 10 minutes at 37°C in a water bath with gently massaging the cord every 2 minutes. The content was then recovered in a 50 mL falcon tube and centrifuged at 1500 rpm for 5 minutes. Endothelial cells isolated after the digestion were resuspended in 5 mL of supplemented Endothelial Cell Media (ECM, ScienCell™ Research Laboratories, USA) containing 10% FBS, 1% penicillin/streptomycin and 5 µg/mL ECGS and plated into a T25 flask (TPP) at 37°C and 5% CO_2. for 24 hours. To maintain and propagate the established primary human endothelial cell culture, confluent endothelial cells are detached by washing twice with sterile PBS and then incubating with 2 ml of Trypsin-EDTA for 2 minutes at 37°C. Next, 2 mL of supplemented medium were added to inactivate trypsin and the endothelial cell suspensions were re-plated in T25 flask (TPP) in 1:3 ratio in 4 ml of supplemented ECM media.

Cells from passage 1 to 6 were used for functional experiments. 48 h before the experiments, cells ($1x10^5$/500 µL of supplemented ECM media) were plated onto 12 mm-round glass coverslips for immunofluorescence, in 24-well plates for flow cytometry and western blot, or in µm pore transwell filters (6.5-mm-diameter, Corning) for trans-endothelial migration assay. Then, medium was replaced with 500 µl fresh complete medium containing either 15 ng/ml TNFα, 15 ng/ml IL-1β or medium alone as control, and the stimulation was allowed for 18 hours at 37°C. Alternatively when indicated, TNFα stimulated HUVEC monolayers were treated for 30 minutes with the proteasome inhibitor 10-100 µM MG-132, calpain inhibitor 10-100 µM ALLN, lysosomal inhibitor 50-100 µM Leupeptin , or DMSO as vehicle control (Sigma, USA). Then, neutrophils were co-incubated onto the activated HUVEC monolayer as described below.

6.8. Co-culture of HUVEC and human neutrophils

To investigate cortactin protein levels in endothelial cells during neutrophil-endothelial interactions, an in vitro co-culture model was performed. $5x10^5$ human neutrophils were resuspended in 500 µL of supplemented RPMI and transferred

to the TNFα stimulated HUVEC monolayer containing $1x10^5$ endothelial cells (Corning Life Sciences, USA). This represents a co-culture containing 5 neutrophils per 1 endothelial cell (5:1 ratio). Neutrophils were allowed to adhere to the HUVEC monolayer for 15 to 30 minutes at 37°C. When indicated, neutrophils were alternatively pre-treated with MG-132 (100 µM), ALLN (100 µM), Leupeptin (100 µM), or DMSO as vehicle control for 2 hours at 37°C, before being co-cultured onto activated HUVEC monolayers. To investigate the effect of NSP inhibitor in endothelial cortactin degradation, neutrophils were alternatively treated with the neutrophil elastase inhibitor sivelestat (100 µM), neutrophil serine proteases PMSF (1 mM) and AEBSF (0.5 mM) for 30 minutes at 37°C before being co-incubated onto activated HUVEC monolayers. Co-cultures were then prepared for flow cytometry, immunofluorescence, or western blot to analyze cortactin protein levels, as described below.

6.8.1. Flow cytometry analysis of co-cultures

The expression of cortactin in endothelial cells after co-cultures, and internalization of NE, CG and PR3 into endothelial cells was analyzed by flow cytometry. After neutrophil co-incubation as indicated above, HUVEC monolayers were washed twice with 500 µL of warm 1x PBS to detach non-adherent neutrophils. The confluent HUVEC cells were detached with 100 µL of TrypLE Express/EDTA (Gibco, USA) and then fixed immediately by incubation in 100 µL of 4% PFA for 20 minutes at RT. After washing twice with 200 µL of cold 1x PBS containing 3% FBS, 0.1% saponin and 5mM EDTA, cell suspensions were blocked using 100 µL of Human TruStain FcX (1:1000) (Biolegend, USA) for 20 minutes at RT. Subsequently, the cells were stained by incubation with AF488-labeled anti-cortactin (clone 289H10, 1:100) or rabbit anti-CG, NE, or PR3 antibodies. For non-labelled primary antibodies, 100 µL of AF-647 secondary anti-rabbit antibody (1:1000) was incubated for 30 min at 4°C. Data were acquired on an FACS Canto II flow cytometer (Becton Dickinson, USA) and analyzed using FlowJo Treestar V10 software (BD Bioscience, USA).

6.8.2. Immunofluorescence staining and confocal microscopy of co-cultures

Cortactin expression, internalization of NSP, and actin dynamics in the co-cultures were analyzed by confocal microscopy. After the co-culture on glass coverslips as described above, medium was removed and replaced with 300 µL of warm 4% PFA for 20 minutes at RT to fix the cells. After washing 3 times with 500 µL PBS + 1% BSA, the fixed cells were permeabilized using 300 µL of 1x PBS containing 0.1% Triton X-100 + 10% FBS for 10 minutes at RT, and then blocked by washing 3 times with 300 µL of PBS + 1% BSA for 5 minutes at RT. The samples were incubated with 50 µL of PBS + 1% BSA containing primary antibodies (anti-cortactin or VE-Cadherin or anti-CG or anti-NE, anti-PR3, or rhodamine-phalloidin) overnight at 4°C. Then, cells were washed 3 times with PBS and incubated with species-specific, fluorescently labelled anti-rabbit-AF647 and anti-mouse AF488, secondary antibodies in PBS for 3 h at RT in the dark. Finally, the coverslips were washed 3 times with PBS and mounted on slides with special ProLong Gold mounting medium with Dapi (Thermo Fisher, USA). Co-cultures were visualized using a confocal laser scanning microscope Leica TCS SP8. Detailed analysis of expression and localization of the indicated molecules were performed using Imaris-Bitplane and ImageJ software.

6.8.3. Western blot

6.8.3.1. Protein extraction

For western blot, $5x10^5$ neutrophils were resuspended in 500 µL of RPMI and incubated for 15 to 30 minutes minutes with the HUVEC monolayer. Then, non-adherent neutrophils were washed off twice with 1x PBS and subsequently 60 µL of Triton-X100 or SDS lysis buffer was added to the co-culture to recover protein extracts.

After treatment or co-culture, HUVEC monolayers were washed twice with 1x PBS. Then, HUVEC monolayer alone, or co-cultures containing HUVEC with strongly adhered neutrophils, were lysed by adding 120 µL of SDS lysis buffer (SDS 1%, 25 mM HEPES, 2 mM EDTA, 25 mM NaF, pH 7.6) in the presence of proteases inhibitor (cOmplete 2x, Roche) and phosphatase inhibitors

(PhosSTOP 2x, Na3VO4 2mM, Roche, Sigma, USA). In some conditions, cells were lysed using Triton Lysis buffer (2% Triton X-100, NaCl 150mM, Tris-HCl pH 8.0 20mM, CaCl2 1mM, Leupeptin 15 µg/ml, PMSF 1mM, Aprotinin 20 µg/ml). Cells were then scraped off in the lysis buffer, and the lysates were sonicated for 3 cycles of 10 seconds at 40% amplitude on ice. Protein extracts were then denaturized by adding 5x Laemmli Buffer (250mM Tris-HCl pH 6.8, 10% SDS, 30% glycerol, 5% β-mercaptoethanol and 0.02% bromophenol blue) and boiling at 95°C for 5 min. Cell lysates were then stored at -20°C until further use.

6.8.3.2. SDS-PAGE

30 µg of protein of each lysate was loaded in 12% SDS-PAGE gels and separated for 2 h at 100 volts. Separated proteins were then transferred to nitrocellulose membranes (Bio-Rad) for 2 h at 220 mA. Membranes were then blocked in TBS containing 0.1% Tween and 5% skim milk for 1 h at room temperature. Afterwards, membranes were incubated with mouse anti-cortactin (clone 289H10, 1:1000) antibody, or rabbit anti-CG, anti-NE or anti-PR3 (1:1000, Invitrogen) antibodies or mouse anti-GAPDH (1,1000 Santa Cruz) antibody as loading control in blocking solution overnight at 4°C with gentle agitation. Blots were then washed 3 times with TBS-Tween 0.1 % for 10 minutes before incubation with anti-rabbit IgG or anti-mouse IgG secondary antibodies coupled to horse-radish peroxidase (HRP) (1:10,000 Santa Cruz Biotechnology) for 1 h at room temperature. Following incubation, blots were washed three times with TBS-Tween 0.01% and revealed using SuperSignal West Femto Chemiluminscent substrates (Thermo Fisher, USA) in the ChemiDoc device (Bio-Rad, USA).

6.8.4. *In vitro* trans-endothelial migration assay

Transwell chambers were used to analyze the effect of the inhibition of neutrophil serine-proteases during trans-endothelial migration. 50,000 HUVEC were seeded into the upper chamber of 5 µm pore transwell filters (6.5-mm-diameter, Corning) pre-coated with 1% gelatin for 30 min at 37°C, and incubated for 48

hours at 37°C. Then, cells were treated with 15 ng/ml TNFα for 12 hours. Before starting the co-culture, 200,000 neutrophils were pre-incubated with 1 mM PMSF, 0.5 mM AEBSF, 100 µM Sivelestat (Sigma, USA) or 2 mg/ml of alpha-1 antitrypsin (Zemaira®, CSL Behring) in supplemented RPMI-1640 media for 30 minutes. The TNF-α was washed off twice from the HUVEC culture using ECM media, and immediately the neutrophils resuspended in 100 µl of supplemented RPMI-1640 medium were added to the upper chamber. The lower chamber was filled with 500 µL of RPMI media containing 100 ng/mL of the chemokine IL-8 as chemoattractant. Neutrophils were allowed to transmigrate for 45 minutes at 37°C. Then, the number of transmigrated neutrophils in the lower chamber were counted using a Neubauer chamber

6.9. Statistics

Statistical analysis was performed using Student's t-test for comparison of two groups, or one-way analysis of variance (ANOVA) with Bonferroni's multiple comparisons to compare between more than two groups (Prism, GraphPad software). Data of each experiment are presented as means ± standard error of the mean (SEM). A value of *$P \leq 0.05$ was considered statistically significant.

VII. RESULTS

7.1. Cortactin is degraded in post-capillary venules during inflammation

Cortactin is known to cluster into ring-like structures surrounding adherent neutrophils to stabilize ICAM-1 mediated neutrophil-endothelial interactions in co-cultures of HUVEC monolayer with human neutrophils[213]. However, spatiotemporal of endothelial cortactin dynamics during neutrophil extravasation have not been studied in vivo. Therefore, we first investigated the expression and localization of cortactin in the microvasculature of mouse cremaster muscles by whole-tissue confocal microscopy. Post-capillary venules (PCVs) were selectively analyzed since neutrophils transmigrate preferentially through these venules. Under basal conditions, cortactin is highly expressed in cremasteric PCVs in both endothelial cells and pericytes. Considerable expression was also detected in extravascular cells of unknown nature (Figure 15A, white asteriks). Using Imaris software, the cortactin signal was specifically analyzed in whole venular endothelial cells and at endothelial junctions, the preferential spots of transmigration, using the PECAM-1 (CD31) signal as a template (Figure 15B). Using this strategy, we found that cortactin was enriched at endothelial junctions in partial colocalization with CD31 (Figure 15C).

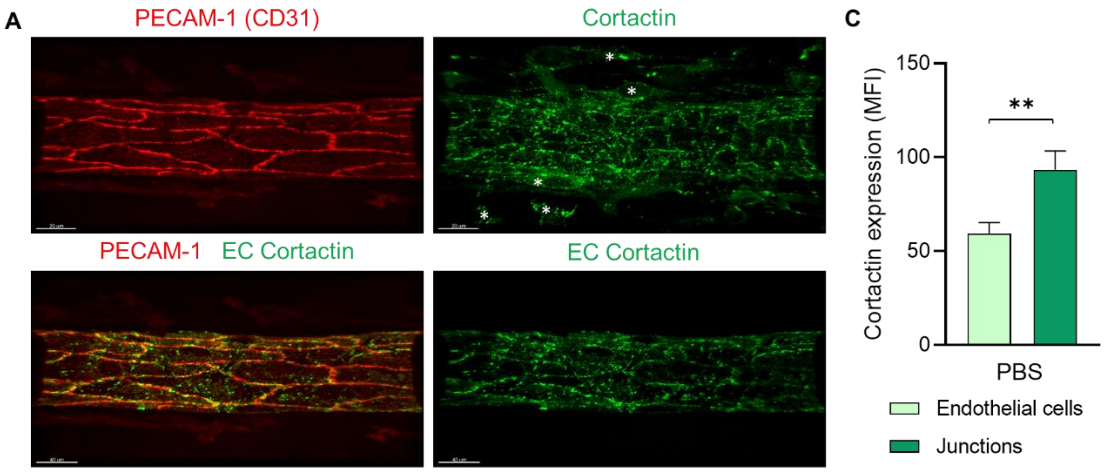

Figure 15. Cortactin is expressed in venular cells and enriched at endothelial junctions. (A) Representative 3D-reconstructed confocal images of cremasteric post-capillary venules (PCVs) immunostained for PECAM-1 (red) and cortactin (green) (Scale bars=20 µm; n=4). Asterisks indicate cortactin in extravascular cells. (B) Cortactin expression in endothelial cells only (lower panel, EC cortactin) was analyzed by Imaris

software using the CD31 signal as template. (C) Quantification of cortactin MFI in whole venular EC and at EC junctions using Imaris (n=4). Data are represented as means ± SEM, *p < 0.05, ∗∗p < 0.01, and ∗∗∗p < 0.001.

To investigate how cortactin expression and localization are altered during inflammation, mice were subjected to acute local inflammation by intrascrotal (i.s.) administration of TNF-α (Figure 16A). Surprisingly, the cortactin signal was significantly reduced over time in venular endothelial cells. Although this response started as early as 30 minutes after TNF-α administration, the cortactin signal was significantly reduced after 2 h and 4 h of TNF- α stimulation compared to the PBS control group. (Figure 16B). These data suggest that cortactin is degraded during inflammation.

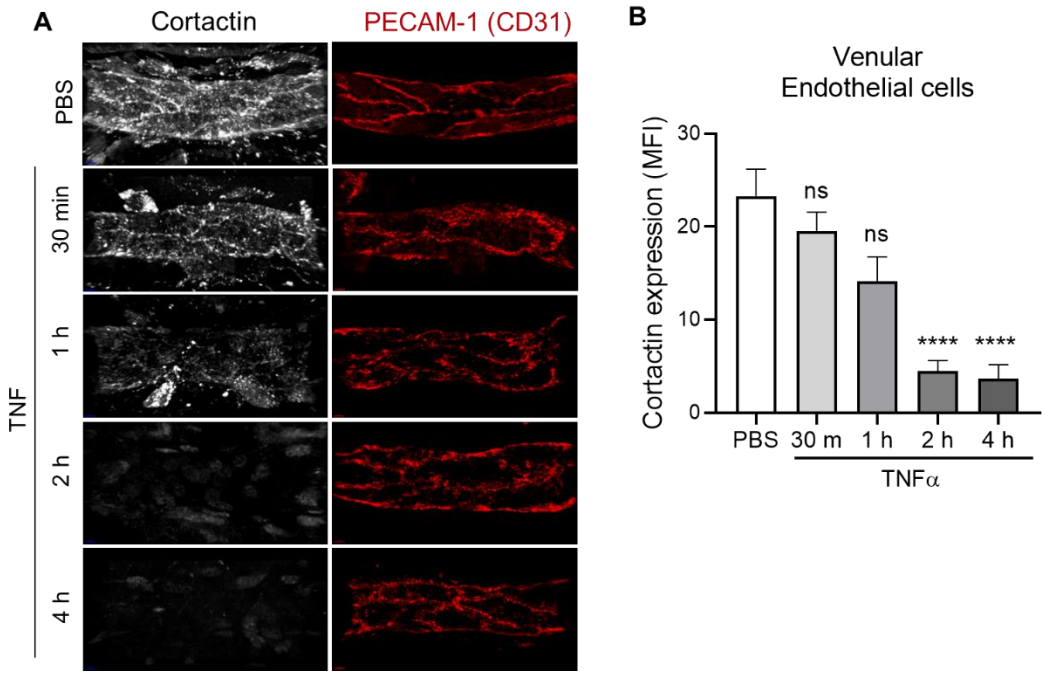

Figure 16. TNFα stimulation induces cortactin degradation in post-capillary venules over time. (A-B) Cremaster muscles were subjected to local inflammation by i.s. administration of TNF-α (300 ng in 400 µl PBS) for the indicated times. PBS was i.s. injected for 4 hours as control. (A) Representative confocal images of inflamed cremasteric post-capillary venules (PCVs) immunostained for PECAM-1 (red) and cortactin (white). (B) Quantification of cortactin MFI in venular EC subjected to local inflammation from the images shown in (A).. Data are represented as means ± SEM; n=3-4 mice/group; ∗p < 0.05, ∗∗p < 0.01, and ∗∗∗p < 0.001; ns, not significant.

7.2. Cortactin is degraded in response to pro-inflammatory cytokines and chemokines, but not histamine.

Next, we analyzed whether these changes in cortactin presence and localization are a TNFα-specific effect. To this end, cremaster muscles were stimulated with either the pro-inflammatory cytokines TNF-α and IL1-β, the neutrophil chemoattractants CXCL1 and LTB4, and histamine. We observed significant reduction of the cortactin signal in PCVs when inflammation was induced by TNF-α (56.62±2.52% of reduction), IL1-β (65.5±6.6%), CXCL1 (68.18±9.88%), or LTB4 (68.18±2.78%) (Figure 17 A-B). By contrast, the pro-inflammatory mediator histamine did not induce significant cortactin degradation, with the levels of cortactin being similar to the PBS control group (10.93±5.81%) (Figure 17A-B). Unlike TNF-α, IL1-β, CXCL1, or LTB4, histamine did not induce neutrophil recruitment in PCVs (Figure 17C). Therefore, we speculated that cortactin degradation was associated with neutrophil extravasation, since it was only evident when neutrophils were recruited.

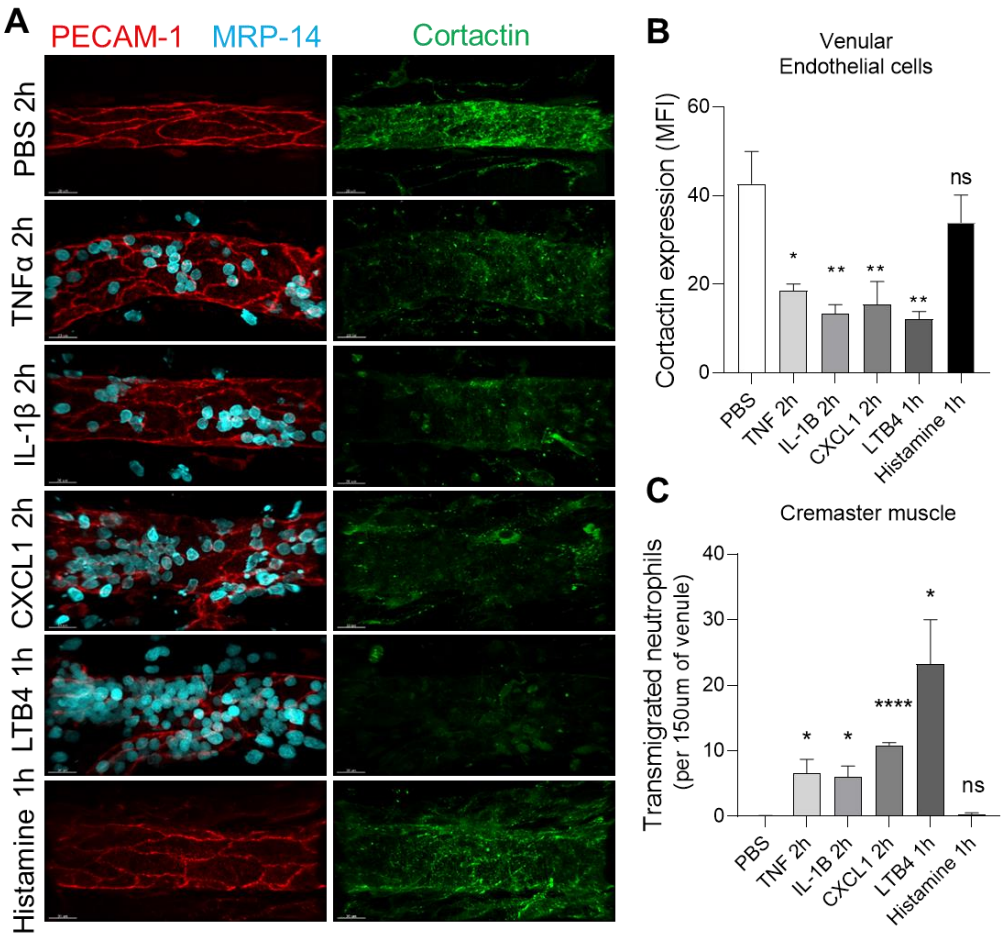

Figure 17. Cortactin is degraded in post-capillary venues in response to inflammatory stimuli that induce neutrophil recruitment. (A-C) Cremaster muscle inflammation was induced by i.s. injection of TNF-α, IL1-β, CXCL1, LTB4 or histamine for the indicated times. PBS was i.s. injected as control. (A) Representative confocal images of inflamed cremasteric post-capillary venules immunostained for PECAM-1 (red) neutrophils (MRP-14, cyan) and cortactin (red) (Scale bars=20 μm). (B) Quantification of cortactin MFI in venular EC subjected to local inflammation from the images shown in (A) using Imaris software. (C) Neutrophil extravasation in cremaster muscles as quantified from the confocal microscopy images shown in (A). Data are represented as means ± SEMs; n=3-4 mice/group; Statistically significant difference between controls and the indicated groups is shown: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$; ns, not significant.

To strengthen this idea, correlation analyses of cortactin degradation and neutrophil extravasation were performed using the quantitative analysis of the confocal images from figure 17. Pooled data from all groups, as well as independent data from each group, showed that those venules with a higher number of transmigrated neutrophils exhibited higher degradation of cortactin, whereas control venules or venules with few transmigrated neutrophils maintained the cortactin signal (Figure 18A-E). Spearman's correlation analysis showed this effect to be significant for all treatment groups, except histamine which did not induce any neutrophil transmigration. These data clearly show that the degradation of endothelial cortactin is associated with the number of transmigrating neutrophils.

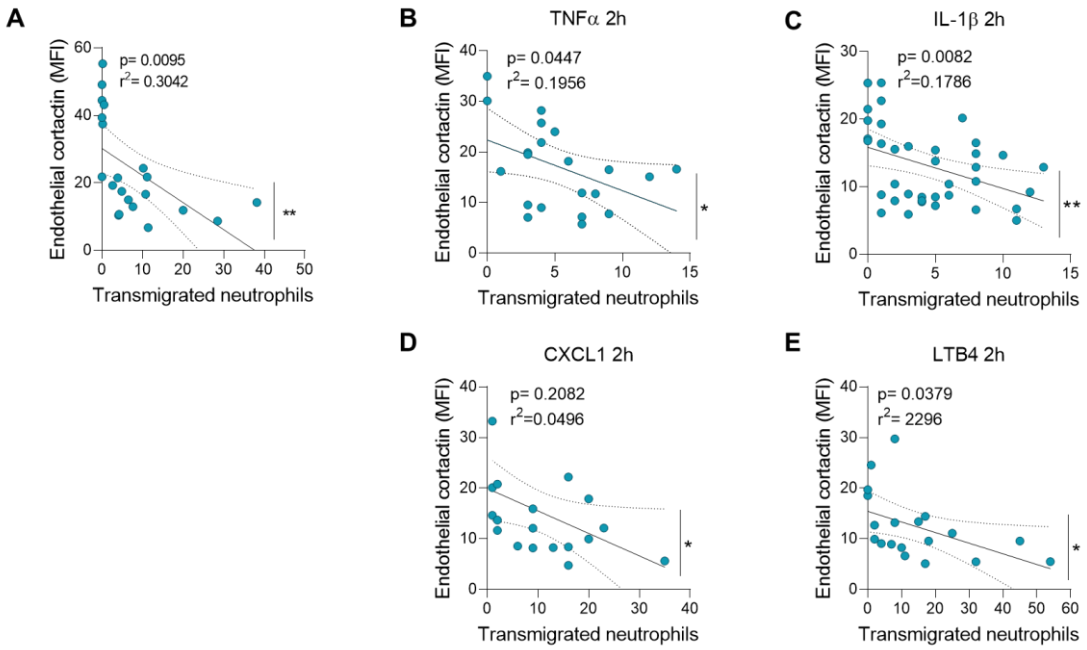

Figure 18. Higher neutrophil extravasation correlates with lower endothelial cortactin. (A) Correlation analysis of the number of extravasated neutrophils and endothelial cortactin MFI at PCVs using the confocal microscopy data from figure 12. Each dot represents the average of each mouse. All groups were included in this analysis (PBS, TNF-α, IL1-β, CXCL1, LTB4, and histamine). (B-E) Correlation of the number of extravasated neutrophils and endothelial cortactin MFI in cremaster muscles stimulated with TNF-α (B), IL1-β (C), CXCL1 (D), LTB4 (E). Each dot represents one PCV analyzed from 3-4 mice per group. Spearman's rank correlation test yielded the p and r^2 values shown in each graph. Statistical significance was considered with a *$p < 0.05$, **$p < 0.01$.

7.3. Cortactin is degraded in PCVs during neutrophil trans-endothelial migration

To confirm the role of neutrophils in the degradation of cortactin, mice were subjected to neutrophil depletion by i.v. injection of α-Gr-1 antibody. 24 h later, acute local inflammation was induced by i.s. administration of TNF-α and cortactin expression at PCVs was analyzed by confocal microscopy. Using this strategy, 92.8±3% of the neutrophil population ($Ly6G^+$ SSC^{high}) was efficiently eliminated from the circulation (Figure 19C-D). The administration of isotype control antibody (IC Ab) or α-Gr-1 antibody (α-Gr-1 Ab) did not affect the expression of cortactin in PCVs from mice i.s. treated with PBS (Figure 19A-B), whereas the mice stimulated with TNF-α and injected with IC Ab exhibited

degradation of cortactin as expected when. Importantly, the degradation of cortactin during TNF-α mediated inflammation was abrogated in neutrophil-depleted mice, with cortactin levels similar to the control group injected with IC AB and PBS (Figure 19A-B). The blockade of cortactin degradation during acute inflammation in the absence of neutrophils further demonstrates that neutrophil recruitment is causing the degradation of cortactin in PCVs.

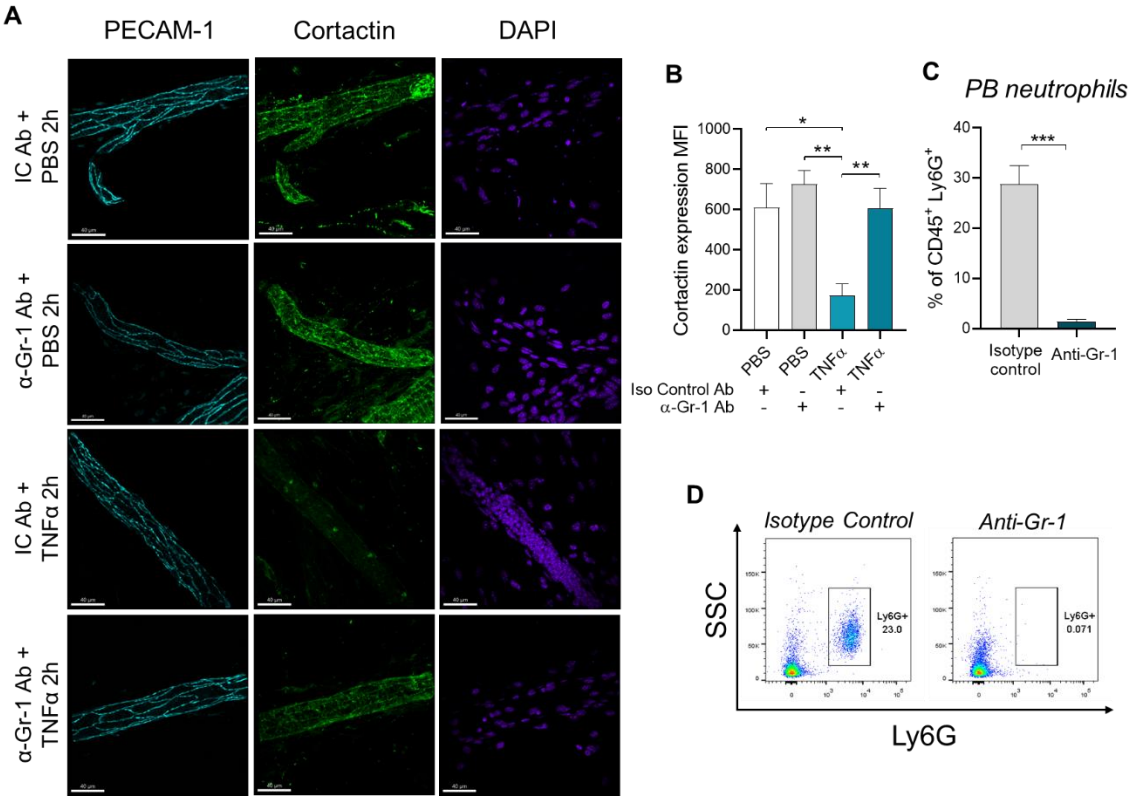

Figure 19. Cortactin degradation occurs during neutrophil recruitment. (A) Representative confocal images of TNF-α (2 hours) stimulated cremasteric PCVs immunostained for PECAM-1 (cyan) cortactin (red), and nuclei (DAPI) from neutrophil depleted mice (α-Gr-1 Ab) or isotype control mice (IC Ab). The control group was treated with PBS and injected with IC Ab or α-Gr-1 Ab (Scale bars= 40μm). (B) Quantification of cortactin MFI in venular EC from the images in (A). (C) Quantification of the percentage of neutrophil depletion in peripheral blood (PB) 24 hours after i.v. injection of isotype control Ab or anti-Gr-1 Ab. The neutrophil population is identified as $CD45^{+}/Ly6G^{+}$. (D) Flow cytometry of PB samples from mice subjected to neutrophil depletion (anti-Gr-1) or treated with isotype control antibody. The neutrophil population is identified as $Ly6G^{+}SSC^{high}$. Data are represented as means ± SEMs; n=3-4 mice/group; Statistically significant difference from controls is shown by $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

7.4. Cortactin is not degraded in HUVEC by proinflammatory cytokines

Next, confluent monolayers of human umbilical vein endothelial cells (HUVEC) were used as in vitro model to investigate in more detail the mechanism of cortactin degradation. Cortactin protein levels were analyzed by western blot in HUVEC monolayers treated with TNFα or IL-1β for 18 hours to induce activation (Figure 20A-B). Both TNFα- and IL-1β stimulated HUVEC did not show cortactin degradation and had protein levels similar to non-stimulated HUVEC (Figure 20A). Pixel intensity analysis demonstrated only non-significant changes of cortactin levels after TNFα or IL-1β stimulation (figure 20B). To assess whether pro-inflammatory stimuli induce any changes in cortactin expression over time, HUVEC monolayers were subjected to a time-course of TNFα stimulation. However, no changes in cortactin expression were observed at any of the indicated time points (Figure 20C-D). In line with our in vivo observations, these results demonstrate that the stimulation of endothelial cells with pro-inflammatory cytokines is not sufficient to induce cortactin degradation.

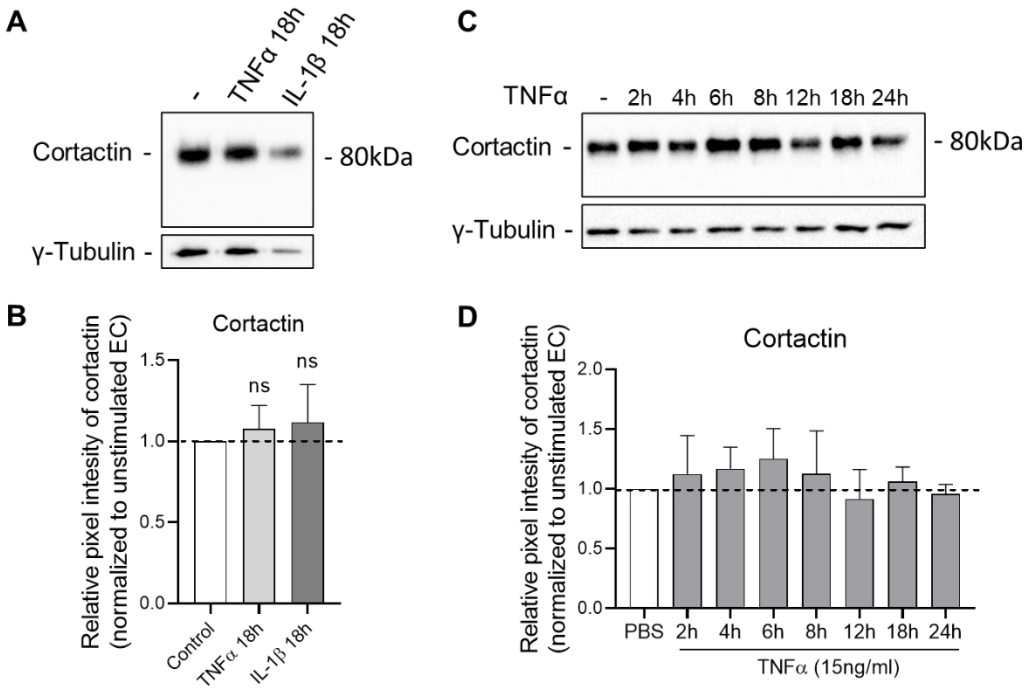

Figure 20. The pro-inflammatory cytokines TNFα and IL-1β do not induce cortactin degradation in cultured human endothelial cells. (A-B) Confluent monolayers of human umbilical vein endothelial cells (HUVEC) were stimulated with TNFα (15ng/ml) or

IL-1β (15 ng/ml) for 18 hours, and cortactin protein levels were analyzed by western blot. (A) Representative immunoblot of cortactin and γ-tubulin (as loading protein control) from HUVEC lysates. (B) Relative quantification of the cortactin band pixel intensity normalized to tubulin expression and unstimulated controls (set to 1, dotted line) (n=4). (C) Immunoblot of cortactin, and γ-tubulin from lysates of HUVEC stimulated with TNFα (15ng/ml) for the indicated times. (D) Quantification of the cortactin pixel intensity normalized to tubulin expression and unstimulated controls (set to 1, dotted line) (n=3). Data are represented as means ± SEMs. ns, not significant.

7.5. Cortactin is degraded in HUVEC upon contact with neutrophils

Our in vivo data strongly suggest that cortactin is degraded upon neutrophil contact with the venular wall (Figure 19). To confirm this observation, neutrophils isolated from human peripheral blood (PB) were incubated with confluent HUVEC monolayers for 15 minutes. Co-cultures were then dissociated using trypLE express/EDTA and cortactin expression was analyzed by flow cytometry in the single cell suspensions. Using this strategy, the expression of cortactin could be easily analyzed in both cell populations present in the co-cultures. Control ECs without neutrophils were identified as cortactin^{high}/CD45^{-}, whereas ECs in the cocultures exhibited a cortactin^{low}/CD45^{-} phenotype (Figure 21A). Neutrophils were identified as CD45^{+}/cortactin^{-} (Figure 21A). Quantitative analysis of the CD45^{-} EC gate showed that EC cortactin was degraded by 78.41±4.98% when neutrophils were co-incubated with endothelial cells (Figure 21B). Of note, cortactin degradation in the co-cultures was similarly induced by neutrophils in both unstimulated endothelial (60.61±4.85% of degradation) cells and TNFα-treated endothelial cells (78.41±4.98% of degradation). Therefore, cortactin degradation is independent of endothelial cell activation and dependent on neutrophil presence. As control of endothelial activation, ICAM-1 expression was analyzed in the cortactin^{+}/CD45^{-} EC population and found to be significantly higher in activated EC than in control EC (Figure 21C). In addition, the number of adherent neutrophils on the EC monolayer was calculated using the flow cytometry data. Although a substantial number of neutrophils adhered to control ECs was found, adhesion was significantly higher on TNF-treated ECs, as expected (Figure 21D). Correlation analysis between the EC cortactin signal and the number of adherent neutrophils showed that the more neutrophils adhered

the lower the cortactin signal was (Figure 21E). This analysis clearly shows a significant correlation between EC cortactin degradation and the number of adherent neutrophils; thus, confirming our in vivo data from PCVs (Figure 18).

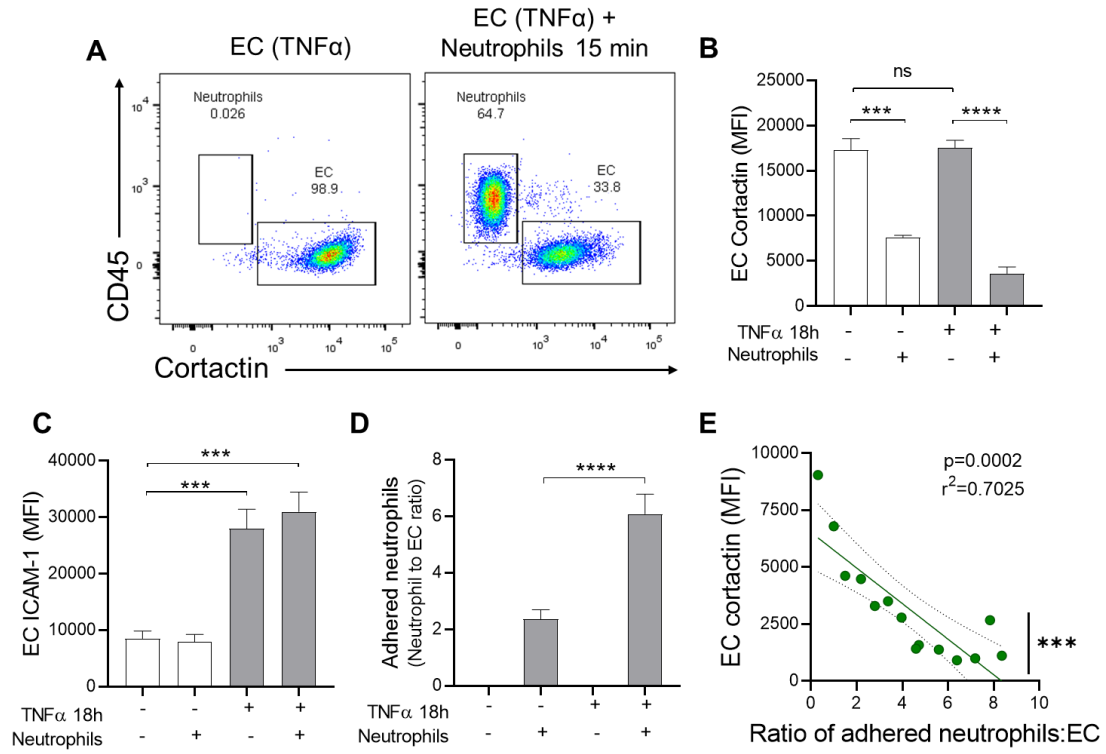

Figure 21. In vitro co-culture of neutrophils with endothelial cells induces cortactin degradation. (A-E) Human peripheral blood neutrophils (1x10^6/ml) were co-incubated with the confluent untreated or pre-treated (TNFα 15ng/ml for 18 hours) HUVEC monolayers for 15 minutes. Co-cultures were dissociated using trypLE express/EDTA and cortactin protein levels were analyzed by flow cytometry. (A) Representative flow cytometry plots of TNFα-treated HUVEC for 18 hours alone (left, cortactin$^+$ CD45$^-$), and co-cultures with human neutrophils (right, cortactin$^-$ CD45$^+$). (B) Quantification of cortactin MFI in the endothelial cells (EC) gate with or without neutrophil co-incubation from the plots shown in (A) (n=4-9 independent experiments). White bars represent co-cultures with unstimulated EC, while gray bars show the EC pre-treated with TNFα (15ng/ml) for 18 hours before adding neutrophils (). (C) ICAM-1 expression in activated and non-activated EC (cortactin$^+$CD45$^-$). (D) Quantification of the number of adherent neutrophils per endothelial cell using the flow cytometry data. (E) Spearman's Correlation analysis of EC cortactin MFI and the number of adherent neutrophils were performed using the flow cytometry data (n=14). Spearman's rank correlation test. Data are represented as means ± SEMs; ∗p < 0.05, ∗∗p < 0.01, and ∗∗∗p < 0.001. ns, not significant.

7.6. Cortactin degradation is not induced by the presence of mononuclear cells

To investigate whether this response is neutrophil-specific, mononuclear cells (MNC) isolated from human peripheral blood were co-cultured with TNFα-stimulated confluent HUVEC monolayers for 30 minutes and 1 h. Cortactin protein levels were then assessed by both flow cytometry and Western blot (Figure 22). As previously observed and used here as positive control, 15 and 30 minutes of neutrophil co-culture resulted in significant cortactin degradation (Figure 22A). However, when MNC were co-cultured with EC, cortactin levels remained similar to the control HUVEC culture without leukocytes (Figure 22A-B). Of note, smaller fragments of cortactin potentially generated by enzymatic processing during neutrophil co-incubation were not identified by western blot (Figure 22B). These data demonstrate that the cortactin is fully degraded without generating truncated cortactin proteins, and that this endothelial response is specifically induced by neutrophils.

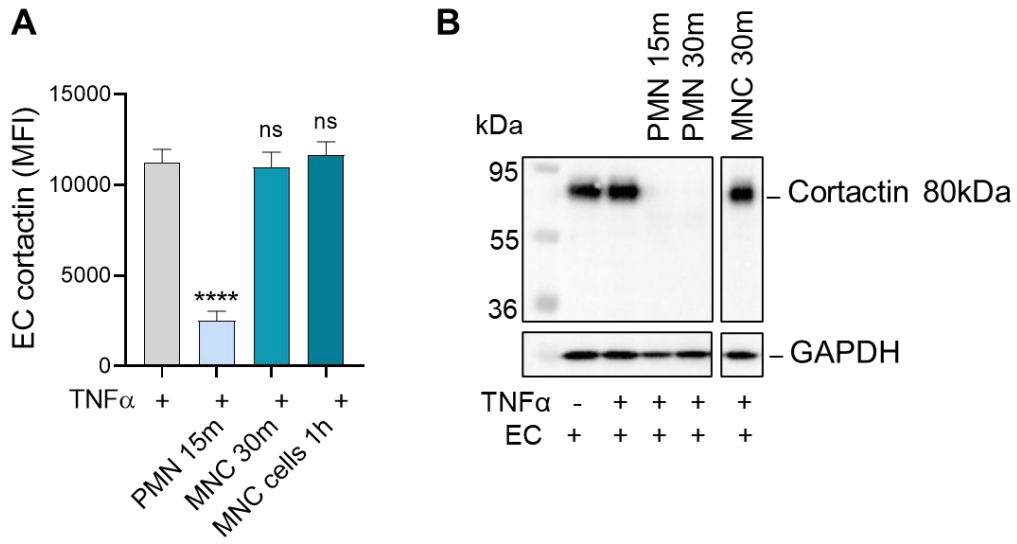

Figure 22. Cortactin degradation is specifically mediated by neutrophils. Confluent HUVEC monolayers were stimulated with 15ng/ml TNFα for 18 hours, then neutrophils (PMN, $1x10^6$/ml) or mononuclear cells (MNC, $1x10^6$/ml) were co-incubated for the indicated times. Cortactin protein levels were analyzed by flow cytometry (A) and western blot (B). (A) Flow cytometry analysis of cortactin MFI in endothelial cells after co-culture with PMN (15 minutes) and MNC (30 minutes and 1 hour). (B) Immunoblot of cortactin and GAPDH as loading control from co-cultures of EC with either PMN or MNC for the

indicated times. Data are represented as means ± SEMs; (n=3-4); ∗p < 0.05, ∗∗p < 0.01, and ∗∗∗p < 0.001. ns, not significant.

7.7. Cortactin localized at the docking site of adherent neutrophils is protected from degradation

Previous studies have shown that cortactin clusters into ring-like structures surrounding adherent neutrophils to stabilize neutrophil-endothelial interactions [213]. To investigate cortactin localization in our model in more detail, co-cultures of HUVEC and neutrophils were analyzed by confocal microscopy (Figure 23). Neutrophils were easily identified by their multilobed nuclei (white squares). Unlike PCVs, HUVEC exhibited homogeneous expression of cortactin throughout the cells including the cytoplasm and plasma membrane, although some junctional regions showed enrichment of cortactin as also seen in PCVs (Figure 23A, white arrows). This localization of cortactin, as well as its expression levels, were similar in both unstimulated and TNFα-stimulated HUVEC (Figure 23A-B). By contrast, when neutrophils were co-incubated with TNFα-stimulated HUVEC, we again observed the strong degradation of cortactin, but we also detected a fraction of cortactin that translocated to the sites of adherent neutrophils, as previously reported [213], that was apparently protected from degradation (Figure 23A, white squares). Quantitative analysis of cortactin signal intensity in these images demonstrated again that the total level of cortactin was again significantly reduced after neutrophil co-incubation, despite these areas of cortactin around adherent neutrophils that remained (Figure 23B). While TNFα-treated HUVEC showed the known increase in actin stress fibers (Figure 23A), the total signal intensity of F-actin did not change after neutrophil co-incubation (Figure 23C). Together, these results indicate that the cortactin pool localized at the sites of neutrophil adhesion is protected from degradation likely to stabilize neutrophil adhesion on the endothelium, whereas cortactin at other locations gets degraded likely to destabilize the actin cytoskeleton and EC junctions.

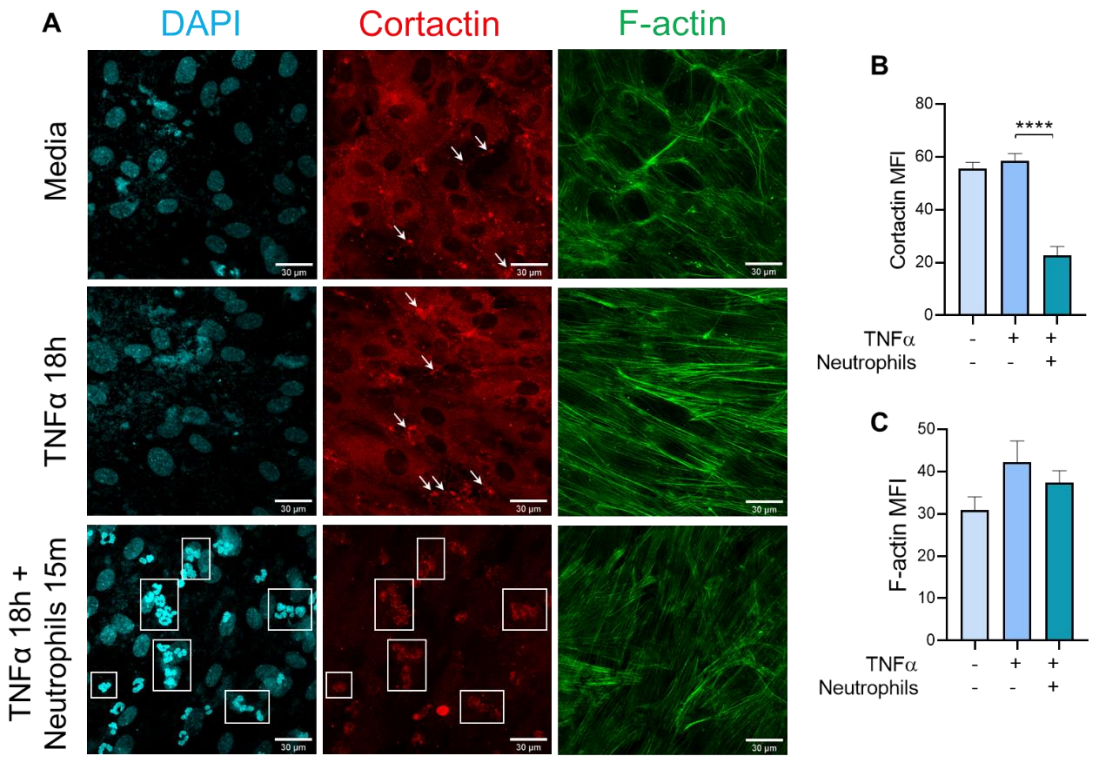

Figure 23. Clustered cortactin at the docking site of adherent neutrophils is protected from degradation. (A) Confocal microscopy images of unstimulated confluent HUVEC monolayers (medium only as control), stimulated HUVEC with TNFα (15ng/ml) for 18 hours, and co-cultures of TNFα-stimulated HUVEC and neutrophils for 15 minutes. Cultures were stained for cortactin (red), nuclei (DAPI, cyan) and actin filaments (phalloidin, green). Cortactin enrichment was observed close to endothelial junctions (white arrows). White squares represent areas with many adherent neutrophils showing cortactin signals protected from degradation (Scale bar=30 µm). (B-C) Quantitative analysis of total cortactin (B) and actin filament (F-actin, C) signal intensities from the images shown in (A). Means ± SEMs; n=3 independent experiments; $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

7.8. Cortactin is not degraded by the endothelial proteasome system, calpain, or lysosomal enzymes

The degradation of cortactin can be mediated by different pathways. For example, deletion of the gen CCM3 causing the inherent diseases cerebral cavernous malformations in human brain endothelial cells has been shown to induce cortactin serine-phosphorylation by ERK1/2 and subsequent degradation of p-cortactin by the proteasome system but not by lysosomes [214]. Moreover, calpain-2 has been shown to mediate the proteolysis of cortactin in fibroblasts[215].

Therefore, the implication of the proteasome system, lysosome, and calpain in the degradation of endothelial cortactin was investigated in HUVEC using pharmacological inhibitors of these molecules. Activated HUVEC monolayers were pre-treated with different concentrations of these inhibitors for 2 hours before co-incubating neutrophils for 15 minutes. Cortactin in co-cultures was then analyzed by flow cytometry. When the proteasome pathway was inhibited in HUVEC monolayers using 10 μM of MG-132 (proteasome inhibitor) or 10 μM PD98059 (ERK inhibitor), the levels of cortactin were similar to the DMSO-treated HUVEC monolayer (gray bars), and no inhibition of cortactin degradation was observed after neutrophil co-incubation (Figure 24A). Higher concentrations of MG-132 (50 μM and 100 μM) were also tested, but cortactin was still degraded as in the DMSO-treated HUVEC monolayer after neutrophil co-incubation (Figure 24B). Calpains were inhibited by pre-treatment of HUVEC with 50 μM and 100 μM of ALLN. However, no changes in the levels of cortactin were observed in HUVEC after the treatments, nor was any inhibition of cortactin degradation observed after co-incubation with neutrophils (Figure 24C). Also, using the lysosomal inhibitor leupeptin, the degradation of cortactin was similar to DMSO-treated HUVEC after neutrophil co-incubation (Figure 24D). Collectively, these results indicate that neither the proteasome system, nor calpains, nor the lysosome degrade cortactin in endothelial cells during neutrophil co-incubation.

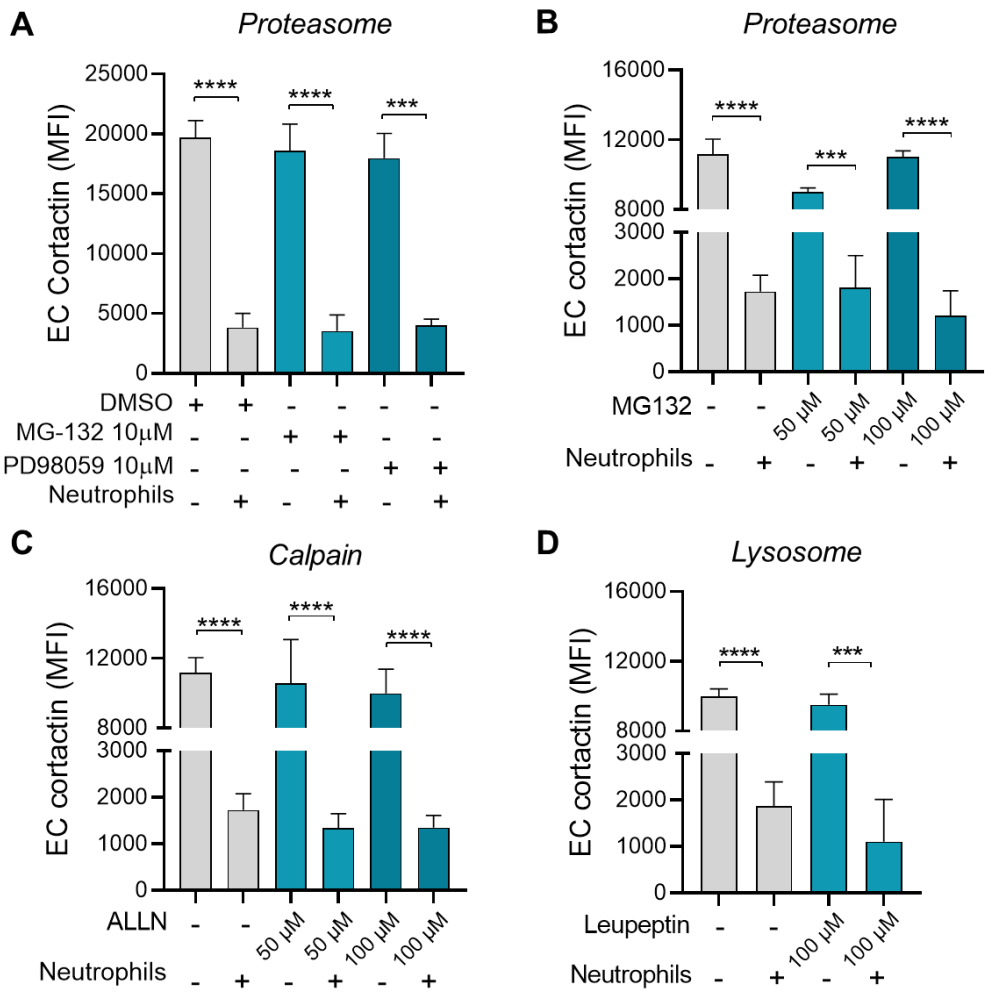

Figure 24. Cortactin is not degraded by the proteasome system, calpain, or lysosomal enzymes. (A-D) Activated (TNFα 15ng/ml for 18 hours) HUVEC monolayers were treated with different pharmacological inhibitors or DMSO as vehicle control for 2 h before co-culture with $1x10^6$ neutrophils for 15 minutes. Cortactin protein levels were then analyzed by flow cytometry. (A) HUVEC monolayers were pre-treated with the proteasome inhibitor MG-132 (10 μM) or the ERK inhibitor PD98059 (10 μM) for 2 h (n=3-4) before co-culture with neutrophils. (B) Activated HUVEC monolayers were treated with the proteasome inhibitor MG-132 (50 and 100 μM) (B), the calpain inhibitor ALLN (50 and 100 μM) (C), or the lysosomal enzymes inhibitor leupeptin (100 μM) (D) for 2 h before co-culture with neutrophils. Cortactin MFI was then analyzed by flow cytometry. Data are represented as means ± SEMs; n=3-4; $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

7.9. Cortactin is degraded by neutrophil proteases

Since the transfer of enzymatically active proteases from neutrophils to endothelial cells has been reported, we then explored whether the degradation of cortactin might be mediated by neutrophil proteases [100]. To investigate this possibility, HUVEC and neutrophils (PMN) were independently lysed using Triton X-100 lysis buffer, and the lysates were immediately co-incubated on ice for 15 minutes and then analyzed by western blot. Co-incubation of EC and PMN lysates also resulted in the degradation of cortactin, similar to the co-culture of neutrophils and EC (Figure 25A), indicating that endothelial cortactin is indeed a target for neutrophil proteases.

To identify the neutrophil protease that degrades cortactin, neutrophils were treated with MG-132 (100 µM), ALLN (100 µM), Leupeptin (100 µM), or a cocktail of all these inhibitors for 2 h before co-culture with activated HUVEC monolayers. Cortactin levels in co-cultures were then analyzed by flow cytometry. When neutrophils were pre-treated with the inhibitors before co-culture, cortactin was still degraded suggesting that EC cortactin is not degraded by neutrophil calpains, lysosomal enzymes or the proteasome.

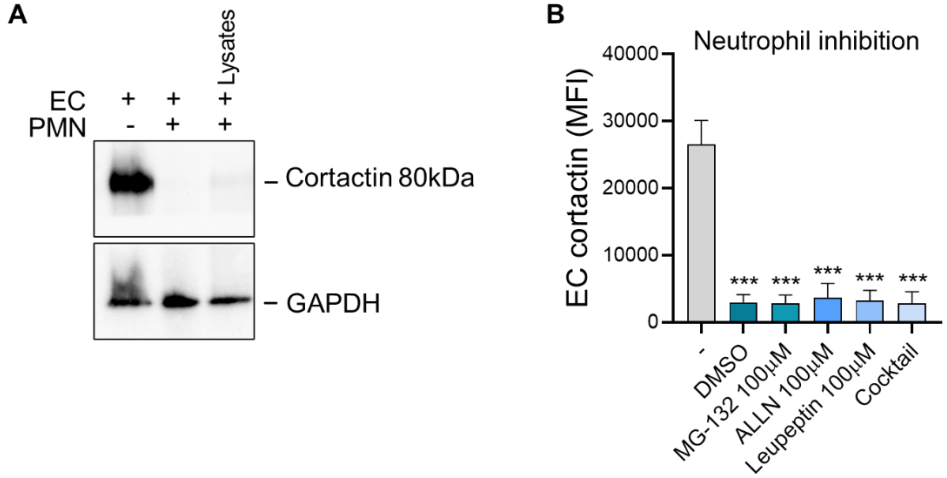

Figure 25. Cortactin is degraded by neutrophil proteases. (A) Immunoblot of cortactin and GAPDH (as loading control) of protein extract from 1x10^5 HUVEC cells alone (EC+ PMN-), or in co-culture with 5x10^5 neutrophils for 15 minutes (EC+PMN+) obtained by adding Triton X-100 lysis buffer. Alternatively, non-cleared extracts from 1x10^{5}neutrophils were immediately added to extracts from 1x10^5 HUVEC and incubated for 15 minutes in ice (Lysates EC+ PMN+). A representative blot from 2 independent

experiments is shown. (B) Confluent HUVEC monolayers were stimulated with TNFα (15 ng/ml) for 18 h) before co-cultures. Neutrophils ($1x10^6$/ml) were pre-treated with DMSO (as vehicle control), or 100 µM of MG-132, ALLN, and Leupeptin, or a cocktail of all these inhibitors at 100 µM each for 2 h before co-incubation with the activated HUVEC for 15 min. Activated HUVEC alone (-) were used as control. Cortactin protein levels were then analyzed by flow cytometry (n=3). Data are represented as means ± SEMs; $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

7.10. Neutrophil serine proteases (NSP) degrade endothelial cortactin

Neutrophil serine proteases (NSP) secreted by activated neutrophils can be internalized by endothelial cells and degrade intracellular proteins leading to changes in endothelial morphology [100]. Therefore, we investigated whether endothelial cortactin is a target for NSP during neutrophil extravasation. To this end, neutrophils were treated with several pharmacological serine proteases inhibitors for 30 minutes before being co-cultured with activated HUVEC. Cortactin protein levels were then analyzed by flow cytometry and western blot (Figure 26). When neutrophil elastase (NE) was inhibited with 50 or 100 µM of Sivelestat (ONO 5046), endothelial cortactin was degraded similarly to endothelial cells incubated with DMSO-treated neutrophils (Figure 26A). By contrast, inhibition with the general NSP inhibitor PMSF at 1 mM significantly reduced the degradation of cortactin compared to endothelial cells incubated with DMSO-treated neutrophils (Figure 26A). Western blot of co-cultures confirmed that endothelial cortactin degradation was partially inhibited when neutrophils were treated with 1 mM PMSF, but not with sivelestat. Of note, similar levels of cathepsin G were observed in all co-cultures, except the control EC without neutrophils indicating that the same numbers of neutrophils were co-incubated and NSP were present in the lysates (Figure 26B). The inhibitory effect of PMSF was concentration-dependent as 2 mM of PMSF almost completely inhibited cortactin degradation after neutrophil co-incubation, with cortactin protein levels being similar to the ones in EC alone (Figure 26C). The degradation of cortactin was also completely abrogated when neutrophils were treated with the serine protease inhibitor AEBSF at 0.5 mM (2482±15 MFI), showing cortactin protein levels similar to EC alone (2463±326.6 MFI) (Figure 26C). Taken together, these

results strongly demonstrated that EC cortactin degradation is mediated by neutrophil serine proteases.

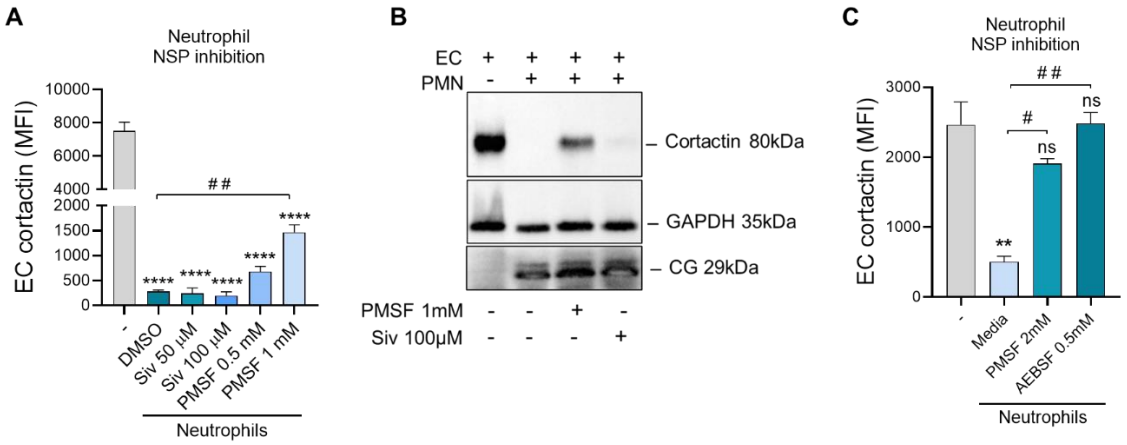

Figure 26. Cortactin is degraded by neutrophil serine proteases (NSP). (A) Confluent HUVEC monolayers were stimulated with TNFα (15ng/ml) for 18 h before co-cultures. Neutrophils (1x10^6/ml) were treated with the neutrophil elastase inhibitor Sivelestat (Siv, 50 – 100 µM) or the general serine proteases inhibitor PMSF (0.5 – 1 mM) for 30 minutes before co-incubation with the activated HUVEC monolayers for 15 min. Control neutrophils were incubated with DMSO as vehicle control. Activated HUVEC alone (-) were used as control of basal levels of cortactin. Cortactin protein levels were then analyzed by flow cytometry (n=4). (B) Immunoblot of cortactin, GAPDH as loading control, and cathepsin G (CG) of protein extracts from co-cultures of neutrophils (1x10^6/ml) with activated HUVEC for 15 min. Neutrophils were treated with PMSF (1 mM) or Sivelestat (Siv, 100 µM) for 30 minutes before co-culture. Control neutrophils were incubated with DMSO as vehicle control. Activated EC alone were used as control of basal levels of cortactin. (C) Neutrophils (1x10^6/ml) were treated with the general serine proteases inhibitors PMSF (2 mM) or AEBSF (0.5 mM) for 30 min before co-incubation with the activated HUVEC monolayers for 15 min. Control neutrophils were only incubated with RPMI medium. Activated HUVEC alone (-) were used as control of basal levels of cortactin. Cortactin protein levels were then analyzed by flow cytometry (n=3). Data are represented as means ± SEMs; $*p < 0.05$, $**p < 0.01$, and $**p < 0.001$. #p < 0.05, ##p < 0.01. ns, not significant.

7.11. NSP inhibition reduced neutrophil trans-endothelial migration

To investigate whether NSP inhibition can affect neutrophil extravasation, in vitro transmigration of human neutrophils treated with the NSP pharmacological inhibitors through HUVEC monolayers was assessed using 5-µm transwell filters (Figure 27). 36.03% of total neutrophils treated with DMSO transmigrated. When neutrophils were treated with sivelestat (100 µM) or PMSF (1mM), trans-endothelial migration was reduced to 17.44% and 12.38%, respectively. By contrast, treatment with AEBSF (0.5 mM) almost completely inhibited transmigration, and only 0.75% of neutrophils transmigrated. These results confirm the previously described role of NSP in neutrophil extravasation and suggest a link between NSP presence, cortactin degradation, and reduced neutrophil extravasation.

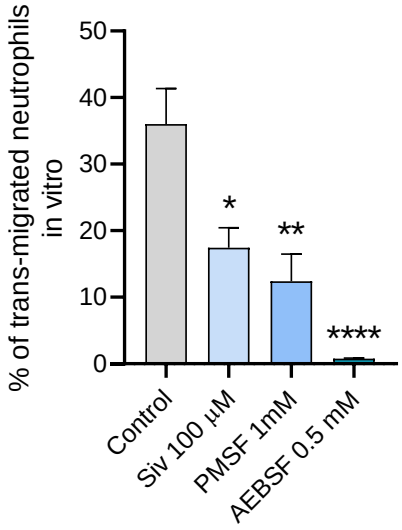

Figure 27. NSP inhibition reduces neutrophil trans-endothelial migration. $2x10^5$ human neutrophils pre-treated with Sivelestat (100 µM), PMSF (1 mM) or AEBSF (0.5 mM) for 30 minutes were allowed to transmigrate through a HUVEC monolayer activated with 15 ng/ml TNFα for 18 h on transwell filters (5-µm pore size) for 1 hour. Control neutrophils were treated with DMSO as vehicle control. The number of transmigrated neutrophils is represented as the percentage of the total cells that transmigrated (n=3 independent experiments). Data are represented as means ± SEM; $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

7.12. NSP alter the architecture of endothelial actin filaments

Internalized NSP have been previously shown to affect actin-cytoskeleton architecture in endothelial cells [100]. Therefore, the organization of actin-filaments was analyzed by confocal microscopy in our co-culture model in the presence of the pharmacological NSP inhibitors. Neutrophils in the co-cultures were identified by their multilobed nuclei stained with DAPI (Figure 28). Cortactin was analyzed in these co-cultures as control of the functionality of the inhibitors. As previously observed, the co-incubation of neutrophils treated with DMSO only led to strong cortactin degradation that was not prevented by Sivelestat (Figure 29A-B). When neutrophils were treated with PMSF or AEBSF, significant inhibition of cortactin degradation was observed compared to co-cultures with DMSO-treated neutrophils with AEBSF even reverting the cortactin signal to control levels (Figure 29A-B).

The organization of actin filaments was observed as typical contractile stress fibers in the TNFα-stimulated HUVEC monolayer alone (Figure 29A). The density of these actin stress fibers was quantified using the threshold method in ImageJ as previously described [216] (Figure 29C). The adhesion of DMSO-treated neutrophils onto the HUVEC monolayer induced even higher actin-stress fiber density compared to the TNFα-activated HUVEC monolayer alone. By contrast, no significant increase in the formation of endothelial actin stress fiber was observed when neutrophils were treated with sivelestat, PMSF or AEBSF (Figure 29A and C). These results suggest that reduced levels of cytosolic cortactin caused by NSP may induce the increased formation of endothelial actin-stress fibers. Of note, this response is associated with NSP as their inhibition results in increased levels of endothelial cortactin and reduced formation of actin-stress fibers. The formation of contractile actin-stress fiber formation is usually accompanied by the opening of junctional contacts. Therefore, the degradation of cortactin by NSP is likely supporting the rearrangement of actin-filaments in EC to mediate the opening of endothelial junctions thus facilitating neutrophil extravasation.

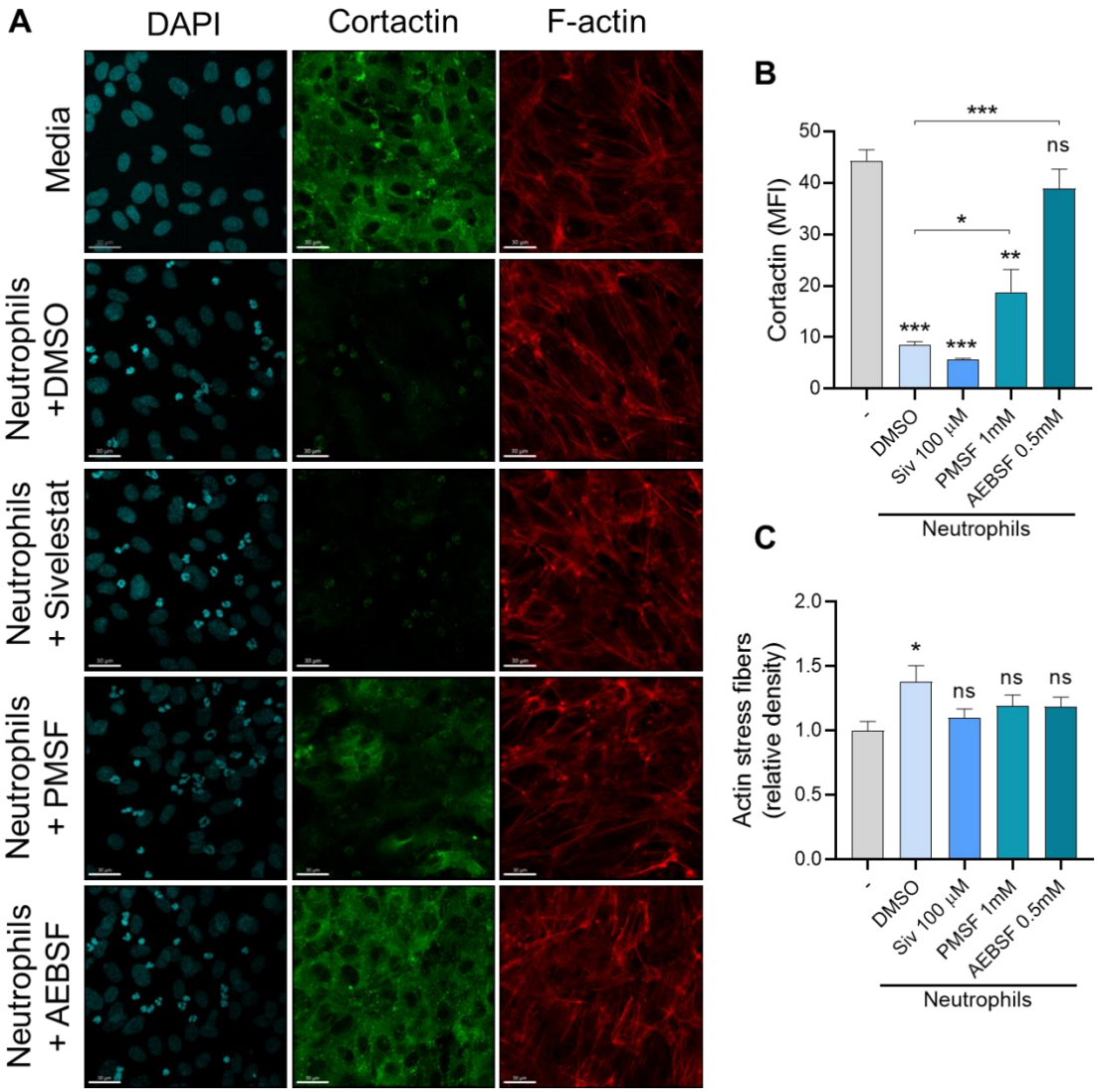

Figure 28. NSP inhibition stabilizes endothelial actin filaments. (A) Confocal microscopy images of stimulated HUVEC monolayer (TNFα 15ng/ml for 18 hours) alone (medium, and in co-cultured with neutrophils ($5x10^5$) for 15 minutes that were treated with DMSO as vehicle control, sivelestat (100 µM), PMSF (1 mM) or AEBSF (0.5 mM) for 30 minutes. Cultures were stained for nuclei (DAPI, cyan), cortactin (green), and actin filaments (phalloidin, green). (Scale bar=30 µm). (B) Quantitative analysis of total cortactin signal intensities from the images shown in (A) using Image J. (C) Quantitative analysis of the density of actin stress fibers using the threshold tool in imageJ. Means ± SEM; n=3 independent experiments; $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

7.13. Neutrophils interacting with endothelial cells release NSP

We have demonstrated that endothelial cortactin is degraded by neutrophil serine proteases. To investigate whether NSP are released during neutrophil-endothelial interactions, cathepsin G was stained in co-cultures of TNFα-activated HUVEC monolayer with neutrophils and analyzed by confocal microscopy (Figure 30). No signal of CG was observed in the HUVEC monolayer alone demonstrating that EC do not express cathepsin G. In the co-cultures, neutrophils were identified by their nuclei and by the expression of cathepsin G. Of note, cathepsin G was not observed exclusively inside the neutrophils, and a considerable signal was detected outside of neutrophils. Using a 3x digital zoom, we detected that cathepsin G outside of neutrophils was particularly enriched at endothelial cell-to-cell contacts. These data suggest that cathepsin G is released from neutrophils during the interaction with endothelial cells. The signal of cathepsin G in the endothelial cells was analyzed in regions of interest (ROI) of 90 μm^2 sizes that were randomly placed in fields without neutrophils to analyze the amount of cathepsin G that was released over the endothelial cells, and thus exclude the cathepsin G signal found within neutrophils. Using this strategy, a significant signal of cathepsin G was observed in endothelial cells after neutrophil co-incubation (Figure 30B). Since CG, NE and PR3 are stored in azurophil granules, and are often found together in extracellular vesicles, it is likely that all NSP are released together during neutrophil-endothelial interactions. Of note, with this strategy it is not possible to identify whether cathepsin G has been internalized or remains bound to the surface of endothelial cells. Therefore, these results indicate that NSPs are released on endothelial cells during neutrophil adhesion. Nevertheless, since NSP internalization was previously demonstrated [100], we believe that in our model, NSP can then be internalized by endothelial cells, degrade cortactin and alter actin-cytoskeleton dynamics.

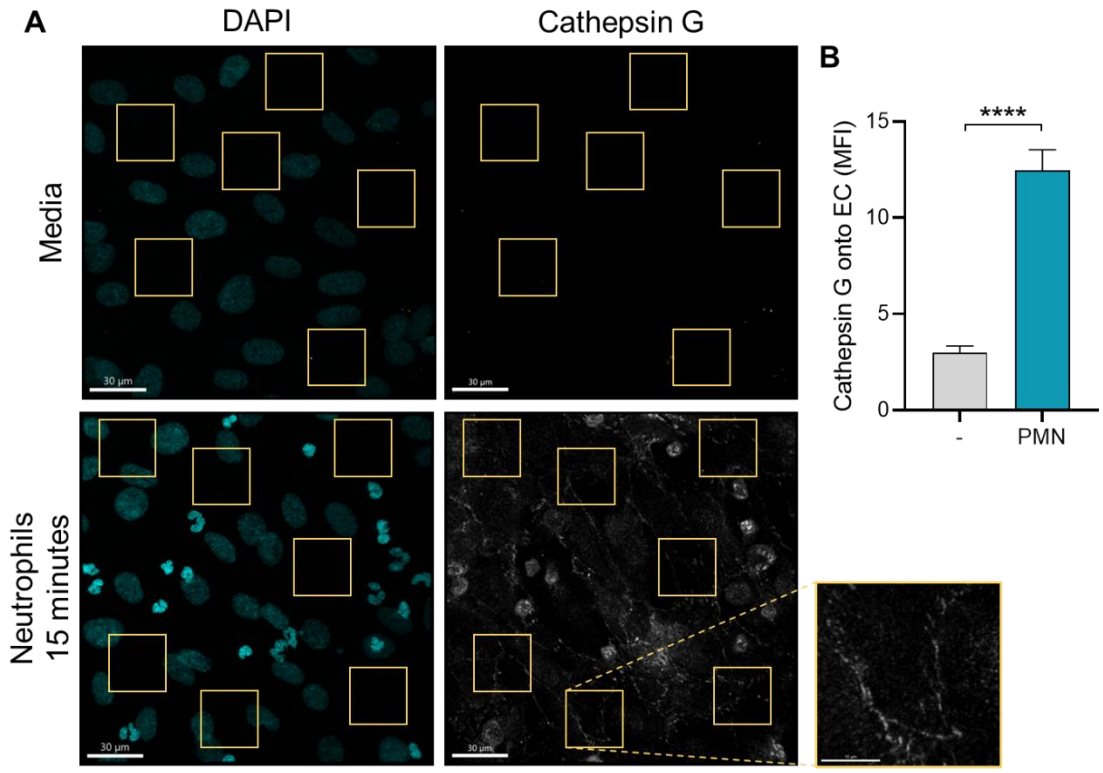

Figure 29. Cathepsin G is released from adherent neutrophils onto endothelial cells. (A) Confocal microscopy images of stimulated HUVEC monolayers (TNFα 15 ng/ml for 18 hours) alone (medium, top), and co-cultured with neutrophils ($5x10^5$) for 15 minutes (bottom). Co-cultures were stained for nuclei (DAPI, cyan), and cathepsin G (gray) (Scale bars=30 µm). (B) The release of CG onto the HUVEC monolayer was quantified by measuring the CG signal intensity in squares excluding neutrophils (yellow, 30 μm^2). At least 6 areas were analyzed using ImageJ. Data are presented as means ± SEM; n=3 independent experiments; $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

7.14. AAT augmentation therapy inhibits cortactin degradation

Because of the high toxicity of PMSF and AEBSF in in vivo models, the specific clinical-grade NSP inhibitor human α1-antitrypsin (AAT) (Zemaira®, CSL Behring) was used to investigate the impact of NSP-mediated cortactin degradation during neutrophil extravasation *in vivo*. AAT (Zemaira) is purified from human serum and has been used clinically to raise the plasma levels of AAT (augmentation therapy) in patients with AAT deficiency and related emphysema, as well as in numerous murine models of inflammation[217–219]. Augmentation therapy consisted of intraperitoneal administration of 2 mg of AAT in C57BL/6

mice. Alternatively, 300 µL of clinical-grade saline solution alone was i.p. injected in C57BL/6 mice as control. 2 h later, 300 ng of TNFα was injected i.s. and incubated for 4 h to induce local cremaster inflammation. Cremaster muscles were then dissected and cortactin protein levels were analyzed by confocal microscopy. Significant cortactin degradation was observed in TNFα-inflamed cremasteric PCV from mice injected i.p. with saline as compared to PBS-treated cremaster muscles that showed normal levels of venular cortactin (Figure 30A-B). Cortactin degradation was abrogated when mice were subjected to AAT augmentation therapy before inducing cremasteric local inflammation even in the presence of intravascular neutrophils (Figure 30A, lower panel). These results confirmed the role of NSP in the degradation of cortactin from PCV and demonstrated that AAT augmentation therapy effectively inhibits cortactin degradation during in vivo inflammation.

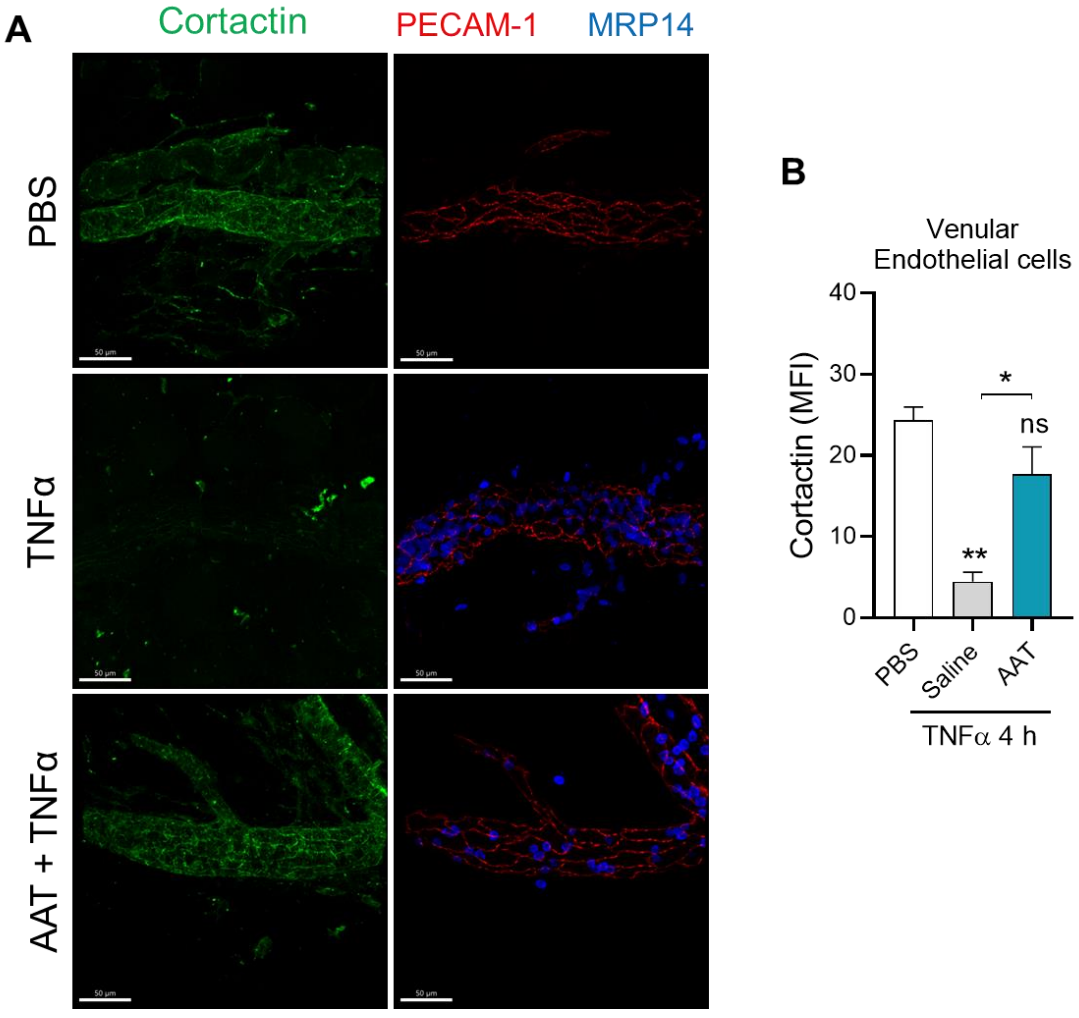

Figure 30. AAT augmentation therapy inhibits cortactin degradation in vivo. (A-B) Mice were injected i.p. with 300 µL of clinical-grade saline solution, or 2mg/300 µL of AAT (Zemaira). AAT was incubated for 2 h followed by 4 h of TNF-α injection (300 ng, i.s.). The control group was treated with 300 µL of saline for 2 h followed by PBS (300 µL) i.s. administration. (A) Representative confocal images of TNF-α-stimulated cremasteric PCVs immunostained for PECAM-1 (red) cortactin (green), and neutrophils (MRP-14, blue) from control mice, or mice subjected to AAT augmentation therapy (Scale bars=50 µm; n=3). (B) Quantification of cortactin MFI in venular EC from the images in (A). Data are represented as means ± SEM; $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

7.15. AAT augmentation therapy also impairs neutrophil extravasation in vivo

Neutrophil extravasation from TNF-α-inflamed cremasteric post-capillary venules was analyzed in mice subjected to AAT augmentation therapy by confocal microscopy. Cremaster muscles treated with PBS did not show neutrophil extravasation, whereas significant neutrophil extravasation was observed after TNF-α cremaster stimulation in mice injected i.p. with saline solution as expected (Figure 31). Importantly, the AAT augmentation therapy significantly reduced the number of extravasated neutrophils from TNF-α-stimulated PCV demonstrating that NSP actively support neutrophil extravasation.

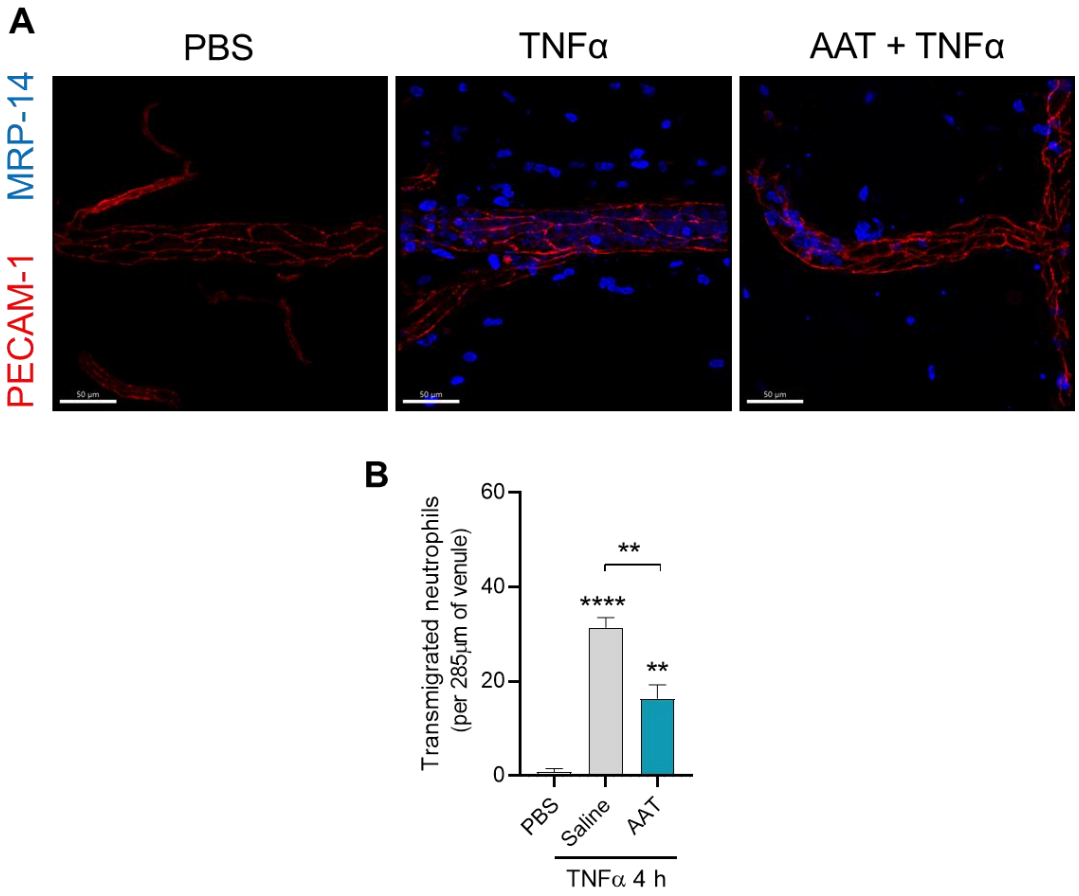

Figure 31. NSP inhibition impairs neutrophil extravasation. (A-B) Mice were injected (i.p.) with 300 µL of clinical-grade saline solution, or 2 mg/300µL AAT and incubated for 2 h followed by 4 h of TNF-α (300 ng) i.s. stimulation. (A) Representative confocal images of TNF-α stimulated cremasteric PCVs immunostained for PECAM-1 (red) and neutrophils (MRP-14, blue) from control mice, or mice subjected to AAT augmentation therapy (Scale bars=50 µm; n=4). (B) Quantification of the number of transmigrated neutrophils per 285 µm of venule from the images in (A). Data are represented as means ± SEM; $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

VIII. DISCUSSION

Much progress has been made in understanding the cellular and molecular events that regulate trans-endothelial migration of neutrophils. However, most studies have focused on the regulatory molecular mechanisms from the neutrophil perspective, while the venular endothelium has long been considered only as a passive barrier that must be crossed. For this reason, the active involvement of the endothelium in the regulation of neutrophil adhesion and diapedesis remains poorly understood. Post-capillary venules must form narrow pores within their intercellular contacts to allow paracellular passage of neutrophils while preventing excessive leakage of plasma proteins into the extravascular tissue. To accomplish this complicated task, tightly regulated endothelial actin-cytoskeleton remodeling is essential to maintain endothelial integrity during neutrophil extravasation[220]. However, the exact mechanisms through which endothelial actin cytoskeleton remodeling supports neutrophil extravasation is incompletely understood. Here, we have demonstrated that the actin-binding protein cortactin undergoes proteolytic degradation by neutrophil serine proteases (NSP) that are transferred to venular endothelial cells during trans-endothelial migration in acute inflammation. Mechanistically, cortactin degradation is associated with increased actin-stress fiber formation, which potentially increases the opening of endothelial contacts, and thus facilitates neutrophil diapedesis. Importantly, alpha-1-antitrypsin (AAT) augmentation therapy inhibited cortactin degradation and reduced neutrophil extravasation *in vivo*. Therefore, we identified cortactin degradation by NSP as a neutrophil-specific mechanism to facilitate diapedesis via increased actomyosin contractility, a response that is inhibited by AAT augmentation therapy.

Cortactin is typically localized at the cell cortex and membrane structures such as lamellipodia and membrane ruffles of migratory cells such as fibroblasts [221]. In cultured endothelial cells, cortactin has been observed in the cytoplasm and at the plasma membrane [214,222,223]. Cortactin translocates to the cell periphery in response to S1P stimulation to stabilize cortical actin filaments and the VE-cadherin junctional complex [214,222,223]. However, the expression and localization of cortactin in a complex tissue in contrast to cultured EC have not been explored

in detail before. Here, we observed enrichment of cortactin at the endothelial junctions in mouse cremasteric post-capillary venules at steady state. Endothelial barrier integrity is largely maintained by the network of circumferential actin filaments that are anchored to the VE-cadherin/catenin complex [224]. Cortactin is known to stabilize actin filaments at its subcellular localizations [221]. Although a direct interaction of cortactin with proteins of the VE-cadherin/catenin complex has not yet been reported, it is likely that the specific accumulation of cortactin at endothelial junctions mediates the stability of the junctional complexes either by direct interaction, or by stabilizing the actin filaments connected to the junctional complexes. This hypothesis is supported by the fact that cortactin deficiency in endothelial cells results in disrupted barrier function and increased endothelial permeability [213,216].

Cortactin has previously been shown to regulate endothelial permeability and neutrophil-endothelial interactions through two distinct mechanisms [213,216]. On the one hand, cortactin deficiency has been associated with increased formation of contractile actin stress fibers via upregulation of the RhoA/ROCK pathway and downregulation of active Rap1, which collectively lead to enhanced endothelial permeability [213,216]. Cortactin controls the activation levels of the GTPase Rap1 that counteracts RhoA-mediated actomyosin contractility to maintain endothelial barrier integrity in basal conditions[213]. On the other hand, despite the weaker endothelial barrier in the absence of cortactin, neutrophils could not take advantage of the open contacts and instead transmigrated less efficiently through cortactin-deficient EC [213]. Mechanistically, this defect was due to defective ICAM-1 clustering into ring-like structures that neutrophils use to firmly adhere to endothelial cells [213]. This study was the first to demonstrate experimentally that regulation of vascular permeability and neutrophil trans-endothelial migration are two separated processes regulated by distinct mechanisms [213]. In this project, we demonstrated that endothelial cortactin is proteolytically processed by neutrophil serine proteases (NSP) that get access into venular EC during in vivo inflammation. Specifically, we observed in our in vitro model that while cytosolic and junction-associated cortactin is almost completely degraded by NSP, cortactin recruited to the docking sites where it is needed to support the firm adhesion of neutrophils on the endothelial cells is protected from NSP-mediated

degradation. The mechanisms underlying this protection from degradation could be that cortactin cleavage sites for NSP are blocked by cortactin interaction with other proteins forming the ICAM-1 adhesion complex [213,225]. Among these molecules, α-actinin and filamin-B have been found to colocalize with cortactin in ICAM-1 clusters [226,227]. However, the exact interaction partner of cortactin in this scenario that blocks NSP cleavage sites remains elusive. In line with previous findings [213], we confirmed that the degradation of cortactin is associated with increased formation of contractile actin stress fibers. We believe that this increased actomyosin contractility facilitates junction opening and neutrophil paracellular diapedesis, which is the preferred route of neutrophil extravasation in the cremaster model [50,228]. This novel scenario with two distinct functional pools of cortactin then perfectly explains the previous apparently contradicting findings that cortactin deficiency increases permeability while reducing extravasation. The cortactin pool required to support neutrophil adhesion to ICAM-1 is protected from degradation and therefore supports neutrophil-endothelial interactions; whereas the cytosolic and junctional cortactin pool that stabilizes junctions is degraded to facilitate junction opening and neutrophil paracellular passage. [213] In line with this new model, numerous studies have demonstrated that the passage of neutrophils through the endothelium via the paracellular route requires the removal of VE-cadherin linked to the actin cytoskeleton via α-catenin. Part of this disassembly occur when neutrophils bind to ICAM-1 clusters which trigger the dissociation of phosphatase VE-PTP from VE-cadherin leading to phosphorylation of VE-cadherin and plakoglobin, and subsequent disassembly of the VE-cadherin complex[229–231]. ICAM-1 clustering also triggers the dephosphorylation of Y731 of VE-cadherin via the tyrosine phosphatase SHP-2, which allows the adaptin AP-2 to bind and enhance endocytosis of VE-cadherin[229]. The opening of adherent junctions is also accompanied by the formation of contractile actomyosin stress fibers that exert pulling forces on junctions. ICAM-1 signaling has also been directly involved in the formation of these contractile actin stress fibers by triggering increased cytosolic Ca^{2+} concentration and activation of RhoA/ROCK signaling [232–235]. Inhibition or absence of kinases that phosphorylate myosin to induce actomyosin contractility (MLCK and ROCK) reduced trans-endothelial migration of both neutrophils and monocytes[227,232,234,236–240]. Therefore, both the signaling downstream of ICAM-1 and NSP destabilize the VE-cadherin complex,

increase actomyosin contractility, and open endothelial contacts in order to facilitate neutrophil paracellular diapedesis.

Neutrophil serine proteases (NSP) represent a highly toxic anti-microbial weapon of neutrophils when they are released into the microenvironment upon infection. However, while the anti-microbial activity of NSP is essential for host survival, its prolonged secretion causes tissue damage. Other non-microbial substrates have been reported as targets for NSP, including host ECM proteins, cytokines, chemokines, and receptors[103]. Thus, based on emerging evidence, NSP are now recognized as important regulators of immune responses. Our findings represent the first report of an endothelial cytosolic protein as a substrate of NSP in acute inflammation *in vivo*. However, the internalization of NSP and processing of intracellular endothelial substrates has been previously described *in vitro* in primary HUVEC cultures and the endothelial cell line ECV304 exposed to recombinant PR3, NE and CG, or neutrophil supernatants containing the three NSP in response to fMLP or anti-neutrophil cytoplasmic antibodies (ANCA) [100,209,210,241]. Proteins from the actin and microtubulin cytoskeleton are among the substrates identified by proteomic analysis [100]. Actin, tubulin1B, vinculin and vimentin were cleaved by these three NSP [100]. However, this study did not identify cortactin as a substrate of NSP using that proteomic strategy, nor other proteins which were previously demonstrated to be degraded by NSP in endothelial cells such as p65 NF-κB and p21/Waf [100,209,210]. This might be due to the conserved analysis algorithms used to avoid false positive substrates, or because of high concentration of purified PR3, NE and CG used in lysates of EC that could lead to total degradation of cortactin and inability to identify the resulting tiny fragments [100]. Our data extend this previous list and included cortactin as an intracellular substrate of NSP in an *in vivo* inflammatory scenario. Whether cortactin can be cleaved by all three NSP or only one of them needs further investigation. The fact that the specific NE inhibitor Sivelestat failed to diminish the degradation of cortactin may suggest that rather PR3 or CG are the enzymes responsible for the proteolytic processing of cortactin. However, given the similarities of substrate specificity of these NSP, it is likely that the inhibition of NE can be compensated by CG and PR3, and therefore only inhibition of all NSP will result in reduced

degradation of cortactin [100]. Thus, we speculate that cortactin is highly susceptible to degradation by all three NSP.

Our findings indicate that neutrophils release NSP in close association with endothelial cells during extravasation and are taken up by the endothelium by a yet unknown mechanism. NSP can be released from neutrophils into EVs or freely by exocytosis of azurophil granules [112]. Therefore, we propose two possible mechanisms of release and internalization: (1) free NSP released by exocytosis are captured and internalized through an endothelial receptor and; (2) secreted EV containing NSP are captured and internalized by EC. *In vitro* conditions mimicking the intravascular microenvironment during trans-endothelial migration demonstrated that neutrophils secrete NSP in response to L-selectin engagement followed by IL-8 stimulation [126,127]. Crosslinking or ligation of CD18 can also stimulate the secretion of elastase [131,132]. Moreover, live imaging of mouse cremaster muscles demonstrated that neutrophils release NE in close proximity to endothelial junctions in response to LTB4 stimulation [133]. Based on this evidence and considering that the first interactions of neutrophils with endothelial cells are mediated by selectins, we speculate that neutrophils rolling on P- and E-selectin encounter TNFα and IL-8, and respond by releasing moderate levels of NSP in the narrow microenvironment formed during neutrophil-endothelial interactions. Moreover, these small compartments formed between neutrophils and endothelial cells could prevent access to endogenous high molecular inhibitors (α1-antitrypsin and α1-antichymotrypsin) found in plasma, and thus NSP are not inhibited in this context and can be internalized by endothelial cells [97,103]. Future investigation will explore whether NSP are in fact secreted during rolling and adhesion, or during diapedesis. Uptake of free NE, PR3 and CG by endothelial cells has been demonstrated [100,209,210]. However, the mechanism of internalization remains undefined. We speculate that free NSP can be captured by endothelial cells through receptors. Previously, a 111 kDa membrane molecule on HUVEC was suggested to mediate the binding of PR3 [242]. The binding of NE and PR3 to endothelial PAR-2 was also recently demonstrated [207 208]. However, there is no evidence to date that these receptors induce the internalization of NSP. Moreover, free, labeled NE and CG (but not PR3) can be taken up by lung cancer cells via clathrin- and dynamin-dependent

endocytosis, but the receptor was not identified [243]. Importantly, they identified that following internalization into early endosomes, NE either traffics to lysosomes, or escapes into the cytosol. Although they did not describe the mechanism, they proposed that NE could catalytically cleave the endosomal membrane to reach the cytosol [243]. Whether NSP enter endothelial cells by the same pathway requires further exploration. On the other hand, NSPs could also be internalized by capturing NSP-containing EVs that are secreted by neutrophils while interacting with endothelial cells [112,180,182,191,192]. Another recent work observed by intravital microscopy the release of EV-like elongated neutrophil derived structures (ENDS) formed by neutrophils rolling on P- and E-selectin [112]. These structures contained, among other neutrophil markers, proteinase 3 and cathepsin G. However, this work did not describe the fate or functional relevance of these structures. Nevertheless, several studies have demonstrated that neutrophil-derived EVs induce pro-inflammatory responses in endothelial cells [116,180,185,187,203]. Therefore, as an alternative hypothesis to the free NSP-receptor mediated internalization, we believe that NSP can also be released in EVs in response to adhesion to selectins and/or ICAM-1. EVs containing NSP could be internalized by some of the previously described mechanisms for EVs such as fusion, receptor-mediated endocytosis or micropinocytosis (Figure 14) [180,182]. Further investigation is needed to identify the receptor by which endothelial cells internalize free NSP or NSP within EVs, and how they reach the cytosol. On top of the discussed mechanisms above, it is possible that NSP or EV-containing NSP may be captured by other neighboring cells at the site of neutrophil transmigration, including pericytes, macrophages, mast cells, and other perivascular leukocytes, that then exert secondary pro-inflammatory effects on the venular endothelium.

We demonstrated that the pharmacological inhibition of NSP by PMSF and AEBSF abrogated the degradation of cortactin in co-cultures of HUVEC and human neutrophils. Similarly, alpha-1-antitrypsin (AAT) augmentation therapy inhibited the degradation of cortactin in post-capillary venules during TNFα-mediated inflammation. Inhibition of NSP by AAT augmentation therapy, as well as by PMSF and AEBSF resulted in reduced neutrophil trans-endothelial migration *in vivo* and *in vitro*. These findings indicate that the degradation of

cortactin by NSP may be part of a neutrophil-specific mechanism to support the crossing through the venular wall. Important roles of NSP in regulating neutrophil extravasation have been previously reported. Similar to our results, numerous studies have shown that the inhibition of NSP reduces neutrophil infiltration to different organs under distinct conditions[59,60,134,193–199,201]. Although our data clearly associate this effect with the inhibition of cortactin degradation and actin-cytoskeletal rearrangement, other extracellular substrates of NSP have been reported to be associated with neutrophil extravasation. For example, proteolysis of VE-cadherin by purified CG and NE or serum from patients with idiopathic inflammatory myopathies containing NSP led to increased endothelial permeability [205,206,244]. In these studies, neutrophil transmigration was favored when VE-cadherin was degraded. Likewise, neutrophil migration was reduced when NSP were inhibited, and this was directly associated with increased presence of VE-cadherin at the endothelial contacts [205,206,244]. Therefore, we propose that the concomitant NSP-mediated degradation of VE-cadherin and cortactin is required to facilitate neutrophil extravasation. Moreover, cleavage of JAM-C by NE was demonstrated in murine cremaster muscles upon I/R injury and LTB4 stimulation, which led to neutrophil reverse TEM [133]. The defective neutrophil extravasation dynamics in the presence of NSP inhibitors have been also associated with blockade of inhibition of venular basement membrane remodeling [59,60,194]. Although the relevance of cortactin degradation in the context of reverse transmigration has not been analyzed here, we believe that NSP-mediated endothelial phenotype shaping regulates neutrophil extravasation dynamics at different levels, and that correct orchestration of all endothelial NSP targets is critical for this important innate immune response.

Alpha-1-antitrypsin (AAT) is the most abundant endogenous NSP inhibitor in plasma and has important roles as anti-inflammatory molecule during severe tissue damage [103]. Patients with AAT deficiency are more susceptible to develop inflammatory diseases, including chronic obstructive pulmonary disease, cirrhosis and colitis [218]. Since AAT is not permeable across cell membranes, AAT antagonizes irreversibly the enzymatic activity of released NSP in the extracellular space including blood to avoid tissue damage caused by persistent release of NSP under inflammatory conditions. AAT administrated in the mice

during augmentation therapy acts mainly by inhibiting intravascularly NSP that are released while neutrophils interact with venular endothelial cells. Thus, the AAT-NSP complex cannot enter the venular endothelial cells and degrade intracellular targets. Importantly, given that AAT significantly reduced neutrophil transmigration *in vivo* during acute TNF-mediated inflammation, our data demonstrate that AAT augmentation therapy induces a general vascular anti-inflammatory response. Numerous previous studies have also reported anti-inflammatory roles of AAT in acute respiratory distress syndrome, colitis, transplantation, and cancer with associated reduced neutrophil infiltration into organs and restored epithelial tight junctions integrity during colitis [245–247].

IX. CONCLUSION

This work demonstrated that both neutrophils and venular endothelial cells are active participants that closely collaborate in regulating neutrophil extravasation. We found that neutrophils release NSP while interacting with the venular endothelium. NSP were captured and internalized by endothelial cells by a yet unknown mechanism to specifically degrade the pool of cytosolic and junctional cortactin. The proteolysis of cortactin is then associated with increased actomyosin contractility and opening of endothelial contacts to allow for neutrophil passage through the venular wall. However, further investigation is required to characterize in detail the molecular mechanisms of NSP internalization into EC, and the cytoskeletal and junctional rearrangements induced by NSP. This novel inflammatory response represents a unique mechanism of crosstalk between neutrophils and EC in the vascular microenvironment at sites of inflammation to support neutrophil trans-endothelial migration. Our finding that NSP inhibition by alpha-1-antitrypsin prevented cortactin degradation and reduced neutrophil extravasation, suggests that cortactin is a potential target and AAT a promising therapeutic strategy to prevent excessive neutrophil extravasation and neutrophil-inflicted tissue damage in inflammatory diseases.

X. PERSPECTIVES

1. To identify which of the NSP is responsible for cortactin degradation.
2. To identify the mechanism of capture, internalization, and fate of NSP in venular endothelial cells.
3. To characterize in detail the endothelial phenotype after NSP internalization, including detailed analysis of actin-cytoskeletal rearrangement and junction organization.
4. To demonstrate that endothelial contacts open after NSP internalization to facilitate transmigration.
5. To investigate by intravital microscopy the effect of AAT augmentation therapy on the different steps of the neutrophil extravasation cascade in post-capillary venules of cremaster muscles inflamed with different inflammatory stimuli.

XI. REFERENCES

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